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YNGVE ZOTTERMAN 80 years

In the 20th of September 1978 YNGVE ZOTTERMAN reaches the age of 80 years. It is with deep feelings of appreciation and gratitude that Acta Physiologica Scandinavica and all those associated with the Journal offer him their warmest wishes for continued happy years and successful activities. YNGVE ZOTTERMAN has the rare privilege to look back this jubilee day on a rich and varied life while still enjoying physical and spiritual health which is only granted a few.

As National Editor for Sweden of Acta Physiologica Scandinavica YNGVE ZOTTERMAN has made invaluable contributions to the development of the Journal. These are not only based on a wide experience in many sectors of physiology but also on his vivid desire to make the Journal representative for Scandinavian Physiology. His own experimental skill combined with an extensive personal knowledge of contemporary physiologists has created the best possible basis for this endeavour. His contacts with a number of leading physiologists has no doubt been stimulated by the close relationship with J. E. Johansson who played an important role in the international organization of physiology and who was once Alfred Nobel's personal assistant in biological research.

YNGVE ZOTTERMAN showed from early years unusual gifts for writing and in parallel with this also for judging the results of other people's scientific production. His own vivid and

lucid style contrasted at times markedly with scientific literature of more conventional type but his enthusiasm was readily aroused when reading papers revealing original ideas, new aspects and significant discoveries.

This spiritual background made YNGVE ZOTTERMAN singularly well prepared for the responsibilities connected with Editorship for a scientific journal. His judgement as selection of suitable referees has been of paramount value for the Journal.

In his capacity as National Editor for this journal he also has had many opportunities to give valuable and stimulating advice to younger authors thereby giving them a lead to new and fruitful aspects of the problem. Sometimes he helped to refresh the memory of more experienced authors as regards the historical development of the problem studied. His interest in the history of science has, as time has passed, led to an accumulation of knowledge in this area which is probably unique in our days. On many occasions his friends and colleagues have enjoyed this invaluable source of—often amusing—information. Many of these treasures have been saved for the future in his book with the witty title "Touch, Tickle and Pain".

YNGVE ZOTTERMAN's contributions to our journal is indeed not restricted to his Editorship however valuable this is and has been. His contributions as author of articles of highest significance spans over a period of some 50 years. In a remarkable series of papers he has covered a number of areas and produced results which have forever connected his name with such fundamental fields as the physiology of single nerve fibres (thesis 1933), the neurophysiology and neurochemistry of the carotid sinus and the carotid body, the physiology of taste, to mention some of the most important ones, each of which would secure him a place in contemporary physiology.

It has been a great privilege for this journal to publish these articles. It is through contributions of this caliber that the reputation of a journal is founded and maintained. To this should be added his outstanding work as organizer of work of vital importance for national nutrition, old age care and his successful work for scientific international symposia will not fail to appear in other contexts. In his indefatigable efforts for the furthering of Scandinavian Physiology the unfailing support from his charming wife should not be forgotten.

On his birthday YNGVE ZOTTERMAN will receive congratulations from all parts of the world. It is with pride and admiration that the Scandinavian Physiologists, through their official publication, add their warm wishes to a great physiologist and friend and express their gratitude for his invaluable work for Scandinavian Physiology.

U S von Euler

Ultrastructural evidence for an innervation of epithelial enterochromaffin cells in the guinea pig duodenum

By

JAN M. LUNDBERG, ANNOCA DAHLSTRÖM, ANDERS BYLÖCK, HÅKAN AHLMAN,
GÖSTA PETTERSSON, INGER LARSSON, HANS-ARNE HANSSON and JAN KEWENTER

Received 29 September 1977

Abstract

LUNDBERG, J. M., A. DAHLSTRÖM, A. BYLÖCK, H. AHLMAN, G. PETTERSSON, I. LARSSON, H.-A. HANSSON and J. KEWENTER. *Ultrastructural evidence for an innervation of epithelial enterochromaffin cells in the guinea pig duodenum*. Acta physiol. scand. 1978. 104: 3-12.

The innervation of the duodenal enterochromaffin cells (E.C.) of the guinea pig was studied at the electromicroscopic level. Pretreatment with 5-OH-dopamine was performed to visualize catecholaminergic (CA) nerves electrically. Near the basement membrane of all examined E.C. in the crypts, bundles of myelinated nerve processes were observed, only partly ensheathed in Schwann cell cover. At least 4 types of processes could be observed. 1) Bundles containing only small clear vesicles, probably cholinergic fibres, 2) bundles with small clear vesicles, and in addition large (200 nm) granules with dense matrix (P-type-fibres), 3) bundles with small electron-dense vesicles, probably CA-fibres, and 4) processes with few vesicles but having the appearance of dendrites. No typical synaptic arrangements were observed, but the minimal distance between the E.C. and the nerve bundles is 150 to 250 nm. Close well within the functional limits of the "autonomic gap". Thus, epithelial E.C. may be influenced by several types of nervous elements, including CA-fibres.

Key words: enterochromaffin cells, guinea pig duodenum, electron microscopy adrenergic nerve terminals, bundles of nerve processes.

The enterochromaffin cells (E.C.) in mammals, such as mouse, rat, guinea pig, cat and man, have been shown to contain serotonin (5-HT) stored in large granules in the cytoplasm (for ref. see Pearse 1969 and Ahlman 1976). In addition to 5-HT the E.C., constituting part of the entero-endocrine cell system, probably also contains some polypeptide gastrointestinal hormones as demonstrated by immunohistochemistry e.g. motilin or substance P (ref. Pearse *et al.* 1974, Helix *et al.* 1976, Polak *et al.* 1976). Thus, the E.C. of the small intestine belong to the so called APUD-group of endocrine cells, which can take up amine precursors, decarboxylate them and store the amine together with the hormonal peptide (cf. Pearse 1969). Also, a common origin of APUD cells from neurally programmed cells of the epithelial origin has been proposed (cf. Pearse *et al.* 1977). Other entero-endocrine

cells in the gut have been shown to store other polypeptides, for instance VIP, gastrin and somatostatin (for ref. see Pearse *et al* 1977).

The functional control of the endocrine cells in the gut, e.g. the E.C. cells, is unknown at present, but suggestions have focused around four main possible mechanisms.

1) Control by changes in the intraluminal milieu of the gut. The shape of the cell, with a thin apical portion carrying villous projections into the gut lumen, is highly suggestive of a receptor function. Instillations of hypertonic glucose and acidification of the duodenum cause degranulation of E.C. and release of 5-HT (e.g. Drapanas *et al* 1962, Fujita and Kobayashi 1971, Kellum and Jaffe 1976).

2) Control by substances circulating in the blood stream. For instance, noradrenaline (NA) infusion to the isolated dog intestine has been shown to cause a release of 5-HT into the perfusate (Burks and Long 1966). Therefore, the suprarenals may hypothetically control the 5-HT release from the E.C. by circulating levels of catecholamines. Also alterations in the level of Ca^{++} in the blood may influence the E.C. (Jaffe, personal communication, 1977).

3) Control by hormones, locally released from neighbouring cells. For instance, gastrin releasing cells of the gastric mucosa have been suggested to be controlled by locally released somatostatin from adjacent cells (*cf* Uvnäs-Wallensten *et al* 1977).

4) Autonomic nervous control. Previous ultrastructural studies on a possible innervation of E.C. have demonstrated the presence of bundles of non myelinated nerve fibres close to the basal lamina of the E.C., but no clear identification of nerve terminal structures was made (Gasbarrini *et al* 1969). However fluorescence histochemical studies have given morphological support for an adrenergic innervation of E.C. Thus, catecholamine (CA) containing varicose fibres have been observed near E.C. in the intestinal crypts (Jacobowitz 1965, Gabella and Costa 1968, Ahlman *et al* 1976a). Also functional evidence for a nervous control of duodenal E.C. has been obtained. Nerve stimulation of the cervical trunk of the vagal nerve caused a decrease in 5-HT content of the duodenum of guinea pig (Hobenleiter *et al* 1971). This decrease in 5-HT could be directly correlated to the E.C. in a cytofluorimetric study which demonstrated a clear decrease in 5-HT content in individual E.C. following stimulation of the cervical vagal nerve in cat (Ahlman *et al* 1976a). Furthermore, denervation experiments and pretreatment with adrenergic blocking agents gave results which strongly suggested that an adrenergic neuronal pathway was involved (Ahlman *et al* 1976b). This is in agreement with previous investigations which reported a release of 5-HT from the small intestine upon stimulation of periaxillary sympathetic nerves or intra-arterial infusion of noradrenaline (NA) (Burks and Long 1966, Tansy *et al* 1971).

Thus, both light microscopical and functional support for a nervous adrenergic control of the duodenal E.C. exists. The purpose of the present study was to investigate the possible presence of a direct innervation of duodenal E.C. with particular respect to adrenergic nerves.

Material and methods

In order to identify adrenergic nerve terminals of the guinea pig duodenum, 5-hydroxydopamine (5-OH) pretreatment was performed (Tranzer and Thomsen 1967). 1 h or 74 h after the i.v. injection of 2

of 5-OH-DA (Chiba 1973), the guinea pigs are anesthetized with Metbital sodium, 30 mg/kg i.p., and perfused-transfixed with 2.5% purified glutaraldehyde in 0.15 M cacodylate buffer pH 7.2, at 20°C. The mesenteric duodenum was then dissected open and immersion-fixed in the same solution for 2 h at 4°C. The tissues of few animals were immersion-fixed only. Thereafter small blocks of the tissues were post-fixed for 90 min in a buffered solution of 2% osmium tetroxide. The fixed tissue blocks were then dehydrated in a graded series of ethanol and embedded in Epon[®]. Semi-thin (1 µm) sections are cut to localize the E.C. at the basal portions of the crypts, based on their content of large irregular osmophilic granules (e.g. Nichols *et al.* 1974). Thereafter, ultrathin serial sections are made of the appropriate areas and contrasted with uranyl acetate and lead citrate.

Results

The E.C. were easily observed in the *chelonoides* due to the presence in the cytoplasm of a large number of irregular granules with a matrix of varying electron-density (cf. Ferreira 1971; Nichols *et al.* 1974; Cheng and Leblond 1974). These granules were in many E.C. very numerous in the basal part of the cell, but also many E.C. with mainly apically situated granules were observed (Fig. 1). The basal parts of the cells rested on the basement membrane of the crypt epithelium (Fig. 2 and 3) while their apices were often extending into the lumen by villous projections (Fig. 1 and 2). Close to the basement membrane bundles of myelinated nerve fibres, incompletely ensheathed in a Schwann's cell cover were frequently observed (Fig. 2 to 5). Observations of serial sections revealed that every examined E.C. cell, and sometimes also neighbouring epithelial cells (Fig. 2), was accompanied by one or more such bundles of nerve fibres. A frequent observation was that the Schwann cell cover was often discontinuous near the base of the E.C. but completely ensheathing the nerve bundles near other intestinal cells (Fig. 2). In many cases typical boutons, filled with apparent synaptic vesicles, were seen close to the basement membrane of E.C. (Fig. 2, 3 and 5). At such places, the Schwann cell cover in most cases was missing (Fig. 3 and 5). The majority of these boutons were filled with clear round vesicles, about 40–45 nm diam. However one or two such boutons in nerve fibre bundles close to the E.C. basement membrane were filled with dark osmophilic granules (about 45 nm diam.), probably due to the uptake of 5-OH-DA in adrenergic nerve terminals (Fig. 1 to 3). No typical synaptic structures could be observed in relation to the boutons near the basement membrane, but the minimum distance between such boutons and the basal membrane of E.C. was 150 to 250 nm (Fig. 3, 5). In some cases, P-type nerve boutons (possibly storing a polypeptide, cf. Baumgarten *et al.* 1970) with large dense vesicles (about 200 nm diam.) were present in the bundles of nerve fibres (Fig. 4).

In the bundles of nerve processes near the E.C. also a vesicle-poor type of fibre was seen (Fig. 4). These processes were of varying diameter but often quite large (2–4 µm). Their cytoplasm contained a few clear vesicles, filaments, microtubules and some mitochondria, but no ribosomes (Fig. 1 and 4). Their appearance was similar to that of dendritic processes observed in the inferior mesenteric ganglion of the cat (Elfvén 1971). In some nerve processes dense material, distributed in a patchlike manner was present just inside the part of membrane which was close to an E.C. Near such membrane specializations, an aggregation of small vesicles was observed (Fig. 4 and 5).

cells in the gut have been shown to store other polypeptides, for instance VIP, gastrin or somatostatin (for ref. see Perno *et al* 1977).

The functional control of the endocrine cells in the gut, e.g. the E.C. cells, is unknown at present, but suggestions have focused around four main possible mechanisms.

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2) Control by substances circulating in the blood stream. For instance, noradrenaline (NA) infusion to the isolated dog intestine has been shown to cause a release of 5-HT into the perfusate (Burks and Long 1966). Therefore, the suprarenals may hypothetically control the 5-HT release from the E.C. by circulating levels of catecholamines. Also alterations in the level of Ca^{+} in the blood may influence the E.C. (Jaffe, personal communication, 1977).

3) Control by hormones, locally released from neighbouring cells. For instance, gastrin releasing cells of the gastric mucosa have been suggested to be controlled by locally released somatostatin from adjacent cells (cf. Uvnäs-Wallensten *et al* 1977).

4) Autonomic nervous control. Previous ultrastructural studies on a possible innervation of E.C. have demonstrated the presence of bundles of non-myelinated nerve fibres close to the basal lamina of the E.C., but no clear identification of nerve terminal structures was made (Gasbarrini *et al* 1969). However, fluorescence histochemical studies have given morphological support for an adrenergic innervation of E.C. Thus, catecholamine (CA) containing varicose fibres have been observed near E.C. in the intestinal crypts (Jacobowitz 1965, Gabella and Costa 1968, Ahlman *et al* 1976a). Also functional evidence for a nervous control of duodenal E.C. has been obtained. Nerve stimulation of the cervical trunk of the vagal nerve caused a decrease in 5-HT content of the duodenum of guinea pig (Hohenlehtner *et al* 1971). This decrease in 5-HT could be directly correlated to the E.C. in a cytofluorimetric study which demonstrated a clear decrease in 5-HT content in individual E.C. following stimulation of the cervical vagal nerve in cat (Ahlman *et al* 1976a). Furthermore, denervation experiments and pretreatment with adrenergic blocking agents gave results which strongly suggested that an adrenergic neuronal pathway was involved (Ahlman *et al* 1976b). This is in agreement with previous investigations which reported a release of 5-HT from the small intestine upon stimulation of periaxillary sympathetic nerves or intra-arterial infusion of noradrenaline (NA) (Burks and Long 1966, Tansy *et al* 1971).

Thus, both light microscopical and functional support for a nervous adrenergic control of the duodenal E.C. exists. The purpose of the present study was to investigate the possible presence of a direct innervation of duodenal E.C. with particular respect to adrenergic nerves.

Material and methods

In order to identify adrenergic nerve terminals of the guinea pig duodenum, 5-hydroxydopamine (5-OH D) pretreatment was performed (Tranzer and Thoenen 1967). 1 h or 24 h after the i. injection of 50

Discussion

The group entero-endocrine (E.E.) cells in the small intestine contain cells with electron-dense granules of varying shape and size, and which store different polypeptide hormones (e.g. review Pearse *et al.* 1977). In the duodenum of most mammals, the majority of E.E. cells are argentaffine (>90%), probably due their content of 5-HT (cf. Nichols *et al.* 1974). Only 5-HT containing E.E. cells are referred to as E.C. cells (cf. Pearse *et al.* 1977). These cells, when studied by immunocytochemical methods, appear to contain in addition to 5-HT also substance P or motilin (see ref. in Pearse *et al.* 1977). E.E. cells containing somatostatin, VIP or neurotensin do not appear to store 5-HT (e.g. Sandler *et al.* 1977; Polak *et al.* 1976), and are mostly distributed in lower parts of the gastrointestinal tract than the duodenum (Polak *et al.* 1976). A few neurotensin cells were, however, observed in the guinea pig duodenum, but only in the villous mucosa, never in the crypts. Thus, the E.E. cells in the crypts of the guinea pig duodenum with large irregular granules, studied in this investigation, are most likely true E.C. cells. Gorgas and Böck (1976) describe various types of granules in E.C. situated in villi and in crypts of the guinea pig duodenum. It is supporting our view that we have studied the E.C. cells that the granules in cryptal E.C. cells were large and irregular (as in our study) rather than small, ovoid or dumb-bell shaped (Gorgas and Böck 1976). Therefore, based on electronmicroscopical characteristics only (without having performed any chromaffin or argentaffin reaction, we consider our cells to represent E.C. cells.

The method to demonstrate catecholaminergic nerve terminals by the 5-OH-DA-method according to Trömer and Thoenen (1967) has been evaluated thoroughly by e.g. Chiba (1973). According to this author the administration of more than 100 mg/kg 5-OH-DA given i.v. or i.p. 1-24 h before death results in a labelling of most adrenergic nerve terminals and of up to 90% of all vesicles in a particular nerve ending. In our studies there was a marked labelling of nerve terminals around blood vessels, indicating that the 5-OH-DA method was working satisfactory in the present experiments. The observation that nerve terminal profiles, labelled with dense cored vesicles, were present in nerve fibre bundles near the basal part of E.C. thus indicates that catecholamine-containing nerve-terminals

Fig. 1 An enterochromaffin cell (EC) from the duodenum of 5-OH-DA-pretreated guinea-pig. The EC contains abundant irregular granules, situated both in the basal part, and in the apical region. Villous processes from the apex of the EC into the gut lumen can be observed (B). Close to the base of the EC bundle of nerve processes (N) is situated. One contains electron dense granules (probably due to uptake of 5-OH-DA) and presumably representing catecholaminergic nerve terminal (→). Other fibres contain small clear vesicles (45 nm diam.). A large vesicle-poor process is also seen, with the appearance of dendritic process (←) (Bar represents 1 µm).

Fig. 2 Oblique section through bundle of nerve processes close to the base of guinea-pig enterochromaffin cell (EC), containing some basal granules. BL = basal lamina of the epithelium. S = Schwann cell cytoplasm (which is discontinuous near the base of the EC). IE = interstitial epithelial cell also bordering the nerve bundle. In the nerve bundle both presumably catecholaminergic (→ stained by the 5-OH-DA pretreatment), possibly cholinergic (← containing small clear vesicles and few larger dense cored vesicles, diam. 70-80 nm) and vesicle-poor dendrite-like (D) nerve endings are present (Bar represents 1 µm).

Fig. 3 The basal part of guinea-pig EC (EC) with dense, irregular granules. BL = the basal lamina of the epithelium. A bundle of nerve fibres is present close to the base of the EC. Both 5-OH-DA-labelled catecholaminergic nerves (→) and profiles with small clear vesicles (B) are present. Note that Schwann cell lamina (S) is discontinuous near the EC. MT = macrophage. (Bar represents 1 µm).



Fig. 13

were present near the basement membrane of E.C. Other types of nerve fibres, containing small (45–50 nm) clear vesicles, alone or together with large granules (about 200 nm) with dense matrix (P-type, cf Baumgarten *et al.*, 1972) were also present close to the E.C.

Innervation of surface-lining epithelial cells has been described to be present also in other organs, e.g. the gall bladder and the stomach. The gall-bladder epithelium, which has a secretory function, is densely innervated by autonomic nerve fibres showing the characteristics of cholinergic nerve-endings. Typical synaptic arrangements were searched for in the study but not observed electromicroscopically. However a functional effect of cholinomimetics on the surface epithelial cells could be demonstrated, suggesting the presence of acetylcholine receptors on the epithelial cells and thus also a nervous control (Axelsson *et al.* 1977). In the stomach, the parietal cells are innervated by cholinergic fibres (cf Hamkert *et al.* 1977). In the intestine, the secreting Paneth's cells have been shown to be innervated by both adrenergic (Gabella and Costa 1968) and cholinergic (cf Ahonen 1973) nerve fibres (also, Lundberg *et al.*, unpublished observations). The E.C. cell, which is also a secretory cell, may thus, in analogy with the gall bladder secretory cells, the parietal cells of the ventricle, and the Paneth's cells, be expected to be innervated by efferent fibres. Since the epithelial E.C. is also an APUD-cell (cf Pearse 1969), and since other APUD-cells, e.g. pancreatic islet cells (Coupland 1958, Libman and Sutherland 1965, Cegrell 1968), and submucosal endocrine cells (Matsuo and Seki 1976) receive an autonomic innervation, it is not surprising to find morphological evidence for an innervation of the epithelial E.C. (cf also Gasharri *et al.* 1969).

In the nerve fibre bundles close to the E.C., nerve processes, essentially devoid of synaptic vesicles, were also present (Fig. 4). These large processes, containing mitochondria, occasional sER-profiles and a few filaments and tubules, might represent dendritic processes, because they have many of the morphological characteristics of dendrites, as described by e.g. Elvén (1971). No ribosomes could be observed in these processes, but if they represent distal parts of dendrites, one would not expect to see many ribosomes. In dendritic processes closer to the cell body ribosomes are observed more frequently (see Taxi 1974). In some cases, such processes contained dense patches attached to the neuronal membrane (Fig. 4.5). Near such a region a collection of vesicular profiles was often present (Fig. 5). Such membrane patches with nearby vesicles have been observed previously in dendritic processes of superior cervical ganglion cells (cf Taxi 1974).

The presence of possible dendritic processes near E.C., suggesting an afferent innerva-

Fig. 4. The basal part of guinea-pig EC (EC) with many irregular electron dense granules. Near the EC bundle of nerve processes, many of which contain few large (diam. about 200 nm) electron dense granules with light halo. These nerve processes have the appearance of P-type nerves, possibly storing polypeptide. A large dendrite-like process, with mitochondria but very few vesicles, is also present (D). Some membrane specializations can be seen in this process (→). MT—microtubules. (Bar represents 1 µm)

Fig. 5. The basal part of guinea-pig EC (EC) with basal granules and ribosomes. N indicates nerve process, EDed with small clear vesicles and some sER-profiles (B-). Membrane specializations can be observed as 2 patches of electron dense material attached to the inner membrane (→). No Schwann cell lamina present. In the EC two large clear vesicles (coated), which may represent endo- or exocytotic vesicles can be seen (←). The distance between the nerve process and the EC is 150–200 nm. (Bar represents 0.5 µm).



Figs. 4 and 5

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The presence of possible dendritic processes near E.C., suggesting an efferent innervation,

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Figs. 4 and 5

E.C. An indirect influence acting via an inhibitory interneuron, is also possible. This hypothetical non-adrenergic interneuron is probably not cholinergic, because atropine did not influence 5-HT release from E.C. in *in vivo* experiments (Ahlman *et al.* 1976 b).

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tion, is very interesting from a functional point of view. Thus, the postprandial vasodilatation in the duodenal mucosa, which can be elicited experimentally by mechanical mucosal stimulation (*cf* Biber *et al* 1971), probably activates an intramural neuronal arch, involving 5-HT as a synaptic transmitter (*cf* Biber 1973 p. 21). The epithelial E.C., having villous processes projecting into the gut lumen, may respond not only to glucose installations and a changed pH in the gut lumen (*cf* Fujita and Kobayashi 1971; Resnick and Gray 1962), but also to mechanical stimulation. This may lead to a liberation of 5-HT and also of the polypeptide (substance P or motilin) which is presumably stored together with 5-HT (*cf* Pearse *et al* 1974, 1977) in the large osmophilic, cytoplasmic granules in the basal end of the E.C., in the same way as calcitonin is stored with 5-HT in sheep parafollicular granules (Atack *et al* 1973). The 5-HT could then possibly activate the suggested dendritic processes, observed in this study in accordance with the arrangement suggested by Biber (1973). It is of interest in this connection to mention that sensory neurons (type II neurons according to Dogiel 1896) of the submucous and myenteric plexuses of the small intestine contain a monoamine oxidase specific for tryptamines (Furness and Costa 1971). The polypeptide, which is presumably released together with 5-HT may of course, also influence dendritic nervous elements, but it may also act as a paraendocrine substance, influencing nearby cell types (*cf* Uvnäs-Wallensten *et al* 1977).

The origin of the various nervous elements observed near E.C. in the present study is still obscure. As concerns the *efferent* systems, the adrenergic nerve terminals presumably originate from adrenergic cell bodies situated outside the gut, e.g. the celiac and/or the mesenteric ganglia. Some of these fibres may also originate in the superior cervical ganglia, since there exists both functional and morphological evidence for a descending adrenergic neuronal pathway from the superior cervical ganglia via the vagal nerves to the duodenal mucosa (*cf* Ahlman *et al* 1976, Lundberg *et al* 1976). The cholinergic nerve fibres may originate from either the submucosal plexus or from the efferent fibres in the vagal nerve. The P type axons, finally, may be processes from polypeptide storing nerve cell bodies which have been observed using immunohistochemical methods in both the *plexus submucosus* and the *plexus myentericus* or from peptide storing neurons in the spinal ganglia (*cf* Hökfelt *et al* 1977). The possible *afferent* nerve processes may hypothetically originate from sensory neurons in the nodose ganglia or possibly from nerve cells in the intramural plexa. According to Dogiel (1896) two types of neurons are present in the submucous plexus. One is probably a motor neuron with one long axon and several short dendrites. The other one (Type II) is probably a sensory neuron with long branched dendrites, ending as sensory receptors. The presumably dendritic processes observed near E.C. in the present study may originate from such sensory cells of the submucous or myenteric plexuses. These sensory cells appear to receive a direct adrenergic innervation while motor cells receive a parasympathetic (probably cholinergic) input (*cf* Jabonero 1953).

The previously demonstrated adrenergic release of 5-HT from E.C., elicited by stimulation of descending CA-fibres in the cervical vagal nerve (Ahlman *et al* 1976a) may operate by direct or indirect nervous influence of the E.C. The direct influence appears to be possible from a morphological point of view since adrenergic nerve terminals have been demonstrated to be present in nerve bundles close to the basal lamina of epithelial

Nervous control of blood flow in the dental pulp in dogs

By

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Abstract

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Pulpal blood flow (PBF) was measured by H_2 -gas clearance in 30 dogs during electrical nerve stimulation, denervation and close intraarterial infusion of noradrenaline (NA). Electrical stimulation of the cervical sympathetic nerve and NA infusion always resulted in decreased PBF. The constrictor effect was reduced by α -blockers, but was never abolished or reversed to vasodilation. Thus, no evidence for β -receptors in the dental pulp blood vessels was found. The fall in PBF produced by cervical sympathetic stimulation could be prevented by cutting the inferior alveolar nerve in the mandibular canal. Stimulation of the cut inferior alveolar nerve in the mandibular canal produced variable pupal flow changes. Both marked increases and decreases were measured. The flow changes during inferior nerve stimulation were unaffected by α -receptor blockade. Atropine reduced the vasoconstrictor response or augmented the dilatory response. Electrical stimulation of the mandibular nerve before its entrance in the canal caused vasodilation, producing sensory sympathetic vasodilation in the dental pulp. Sympathetic denervation had little or no effect on PBF. Total cutting of the inferior alveolar nerve in the canal increased PBF in lower doses from 0.19 to 0.219 ml/min g. The experiments show that pulp blood vessels of the dog may be controlled by both vasodilator and vasoconstrictor nerve fibres.

Despite the abundant histological evidence for the presence of different nerve fibres terminating on or in close contact with dental pulp vessels, little information is available on the vaso-regulatory function of these fibres. Arwill (1958) found two types of unmyelinated nerve fibres in the human dental pulp, but only one of them was supposed to be of autonomic origin and located in the vascular wall. A close connection of sympathetic fibres with pupal vessels and an acetyl-cholinesterase-positive nerve fibre network on some blood vessels have been demonstrated by Pohjo and Anttila (1968).

Avery *et al.* (1971) noted significant drop in specific cholinesterase after resection of the inferior alveolar nerve, whereas removal of the cervical sympathetic nerve resulted in an increased cholinesterase reaction throughout the pulp. The presence of cholinergic nerve fibres in the pulp has also been supported by Avery *et al.* (1974) and Kukletová *et al.* (1968).

A number of investigators have concluded that stimulation of the cervical sympathetic nerve or infusion of catecholamines caused a reduction of blood flow in the dental pulp (Taylor 1950, Ogilvie 1969, Edwall and Kindlová 1971, Beer *et al.* 1974), but information concerning the type of adrenergic receptors is sparse and partly contradictory (Weiss

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TONDER, K. H. and G. NASS. Nervous control of blood flow in the dental pulp in dogs
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Pulpal blood flow (PBF) was measured by H_2 -gas clearance in 30 dogs during electrical nerve stimulation, deservation and close intraarterial infusion of noradrenaline (NA). Electrical stimulation of the cervical sympathetic nerve and NA infusion always resulted in decreased PBF. The constrictor effect as reduced by α -blockers, but it was never abolished or reversed to vasodilation. Thus, no evidence for β -receptors in the dental pulp blood vessels was found. The fall in PBF produced by cervical sympathetic stimulation could be prevented by cutting the inferior alveolar nerve in the mandibular canal. Stimulation of the cut inferior alveolar nerve in the mandibular canal produced variable pulpal flow changes: Both marked increases and decreases are measured. The flow changes during inferior nerve stimulation were unaffected by α -receptor blockade. While atropine reduced the vasoconstrictor response or augmented the dilatory response, Electrical stimulation of the mandibular nerve before its entrance in the canal caused vasodilation, proposing sensory adrenergic reflexes in the dental pulp. Sympathetic deservation had little or no effect on PBF. While cutting of the inferior alveolar nerve in the canal increased PBF in lower canine from 0.139 to 0.219 ml/min. g. The experiments show that pulp blood vessels of the dog may be controlled by both vasodilator and vasoconstrictor nerve fibers.

Despite the abundant histological evidence for the presence of different nerve fibers terminating on or in close contact with dental pulp vessels, little information is available on the vaso-regulatory function of these fibers. Arwäll (1958) found two types of unmyelinated nerve fibers in the human dental pulp, but only one of them was supposed to be of autonomic origin and located in the vascular wall. A close connection of sympathetic fibers with pulpal vessels and an acetyl-cholinesterase-positive nerve fibre network on some blood vessels have been demonstrated by Pohjo and Anttila (1968).

Avery *et al.* (1971) noted a significant drop in specific cholinesterase after resection of the inferior alveolar nerve, whereas removal of the cervical sympathetic nerve resulted in an increased cholinesterase reaction throughout the pulp. The presence of cholinergic nerve fibers in the pulp has also been supported by Avery *et al.* (1974) and Kukletová *et al.* (1968).

A number of investigators have concluded that stimulation of the cervical sympathetic nerve or infusion of catecholamines caused a reduction of blood flow in the dental pulp (Taylor 1950 Ogilvie 1969 Edwäll and Kindlová 1971 Boer *et al.* (1974), but information concerning the type of adrenergic receptors is sparse and partly contradictory (Weiss

et al 1969 Edwall 1971 Bolvin *et al* 1974) The sympathetic postganglionic fibers arising in the superior cervical ganglion reach the dental pulp of the lower teeth by travelling as a plexus around the carotid artery through the mandibular nerve and finally through the inferior alveolar nerve (Christensen 1940, Ogilvie 1969) However previous studies of stimulation of the mandibular or inferior alveolar nerve have given conflicting results in regard of blood flow Thus Kroeger (1968) stimulated the distal cut end of the inferior alveolar nerve and recorded an increase in intrapulpal pressure (opposite of the response to sympathetic stimulation) while stimulation of the mandibular nerve in the canal produced no change in pulp blood flow measured by impedance plethysmography (Neidle and Liebman 1964) Bishop and Dorman (1963) reported that electrical stimulation of the distal end of the cut mandibular nerve produced vasoconstriction, while sectioning of the mandibular nerve resulted in an increased blood flow in the mandibular artery This increase in flow might result from removal of sympathetic vascular basal tone or mechanical stimulation of vasodilator fibers. However experimental evidence for nervous vasodilation in the pulp caused by either sympathetic or other nerve fibers is lacking.

The purpose of the present investigation was to study pulp blood flow changes during electrical nerve stimulation and denervation in order to determine whether the various nerves contribute vasomotor efferent fibers, either dilator or constrictor to the blood vessels in the dental pulp in dogs. The cervical sympathetic, mandibular and inferior alveolar nerves were stimulated and attempts were made to identify the vasoactive fibers by means of receptor blocking drugs.

Methods

Experiments were performed on 30 adult healthy mongrel dogs of different sizes and sex. The animals were anesthetized by intravenous injection of Nembutal (sodium pentobarbital), 25 mg per kg b.w. A tracheal cannula was inserted and the jaw immobilized. A femoral artery and vein were exposed and cannulated, the vein for infusion and the artery for continuous recording of systemic arterial pressure with a Hewlett Packard transducer and recorder.

Blood flow in the dental pulp of the canine teeth was estimated by measuring local H_2 -gas clearance with platinum electrodes implanted in the pulp 2-8 days previously (Tonder and Aukland 1975). H_2 -gas was supplied by inhalation of about 5% H_2 air for 3-15 min, providing stable concentration in the pulp. Pulpal blood flow (PBF) in ml/min per g tissue was calculated from the rate constant of the 11 deoxygenation curves obtained after stopping H_2 -gas inhalation. The rate constant, k , was obtained by plotting the electrode current caused by oxidation of $H_2(O_{10})$ on a logarithmic scale against linear time scale as previously described (Tonder 76). The coefficient of variation for subsequent measurements was with this method 3.1% (Tonder and Aukland 1975).

The cervical sympathetic, the mandibular or the inferior alveolar nerve was dissected free for electrical stimulation on the side where pulp blood flow was measured. In experiments with sympathetic stimulation the common carotid artery was exposed together with the sympathetic nerve, and blood flow in the common carotid artery (CCBF) was recorded with a Nycotron electromagnetic flowmeter. The stimulating electrode was placed caudal to the superior cervical ganglion and preganglionic stimulation was performed.

The sympathetic nerve in the neck of the dog runs together with the vagus in the common vago-sympathetic trunk and careful dissection of the trunk was necessary to separate the sympathetic portion. Identification of the sympathetic nerve was made functionally by observing the ipsilateral pupil and blood flow in the common carotid during stimulation. The sympathetic nerve was cut proximal to the stimulation point to exclude spreading of the stimulus via afferent fibers.

The mandibular nerve was exposed through intraoral incision, dissected free and cut one cm before its entry into the mandibular foramen. The inferior alveolar nerve was surgically exposed in the mandibular

canal by removal of buccal segment of cortical bone distal to the second premolar. All nerves were stimulated in efferent direction using bipolar silver-silver chloride electrode with monophasic square wave pulses (Green Model). The parameters of the stimulation were: pulse duration 0.5–6 ms, frequency 1–20 Hz and voltage 4–30 V. The stimulation periods were of 4 to 12 min duration.

In those experiments where the effect of noradrenaline (NA) was tested the external carotid artery was dissected free through an incision behind the jaw on the side of the extended tooth. The lingual artery was cannulated in retrograde direction for NA infusion. An electromagnetic flow probe (Nycotron, Oslo) with diameter 2.5 mm was placed on the external carotid, few mm distal to the infusion site.

The following drugs were used: Noradrenaline (0.01–5 µg/min), atropine (sulphate 1 mg/min) and phencyclidine (50 µg/min). The drugs were diluted in 0.9% saline and infused at rate of 1 ml/min. PBF was measured during control conditions alternating with periods of nerve stimulation and/or drug infusion.

Pulpal vascular resistance (R) is calculated as arterial pressure in mmHg divided by PBF in ml/min g, giving the resistance unit mmHg min g/ml. For comparison of different experiments, the values of PBF, AP and R were calculated in per cent of the averaged control values obtained immediately before and after the experimental periods.

Results

Control measurements

Control measurements were usually made before and after stimulation and drug infusion, and their average was used for comparison with the intervening experimental period. Mean PBF measured in the canine teeth in 30 dogs during control conditions was 0.166 (SE 0.003) ml/min g (83 measurements) and control systemic pressure averaged 115 mmHg. Usually arterial pressure and pulpal blood flow remained fairly constant even in experiments of several hours' duration.

Effect of cutting the inferior alveolar nerve

Measurements of PBF in the lower canine were performed before and after cutting the ipsilateral inferior alveolar nerve in the canal in 13 dogs. Compared to recordings after surgical exposure of the nerve, denervation caused a rise in pulpal blood flow of ipsilateral lower canine in 10 dogs. In three dogs, cutting of the nerve had no effect on PBF (Fig. 1). Control measurements before surgical exposure of the nerve (5 dogs) demonstrated that surgery had no consistent effect on PBF. On average, PBF in the ipsilateral lower canine rose from 0.159 (SE 0.017) ml/min g to 0.219 (SE 0.027) ml/min g after denervation. Calculated pulpal resistance fell on average by 23 per cent, but repeated measurements during the first hours after denervation showed that this fall in resistance might be transient (Fig. 1). Simultaneous recordings of PBF in the ipsilateral upper canine showed no consistent change after cutting the inferior alveolar nerve.

Inferior alveolar nerve stimulation

Efferent stimulation of the cut inferior alveolar nerve in the canal had inconsistent effect on blood flow in the ipsilateral lower canine, as shown in Fig. 2 (left panel). Stimulation caused no change in systemic arterial pressure, while PBF ranged from 35–153 per cent compared to control measurements. In a total of 27 measurements (16 dogs), PBF fell to 91% (SE 6) of control and calculated vascular resistance rose to an average of 121% (SE 8) of control (Fig. 3, left panel). Simultaneous measurements of PBF in the ipsilateral

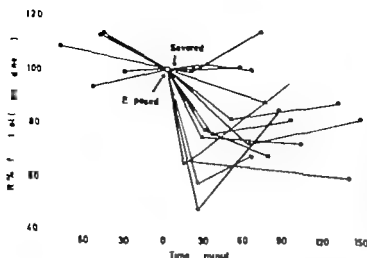


Fig. 1 Effect of cutting the inferior alveolar nerve on pulpal vascular resistance (R) in ipsilateral lower canine tooth. — Open circles: Control measurements. Closed triangles: First measurements after denervation. Closed circles: Second measurements after denervation.

upper canines (12 dogs) showed that blood flow here remained unaltered during the stimulation periods.

In 7 dogs (9 measurements) phenoxybenzamine (7 mg/kg b.w.) was given i.v. prior to stimulation. As shown in Fig. 3 right panel, alpha-adrenergic blockade did not significantly change the effect of stimulation of the inferior alveolar nerve. Control blood flow in the lower canine averaged 0.156 (SE 0.013) ml/min/g compared to 0.165 (SE 0.022) ml/min/g during stimulation and calculated pulpal resistance showed no consistent change ($p < 0.05$).

Intravenous infusion of atropine in 6 dogs consistently altered the effect of stimulation on PBF in the ipsilateral lower canine. Compared to measurements during nerve stimulation before atropine administration, blood flow in the lower canine was higher during stimulation after atropine infusion. This was a surprising but consistent finding in recordings in 6 dogs. Thus, in all 6 expts. pulpal vascular resistance (R) was lower during stimulation after (77% of controls) than before (115% of controls) infusion of atropine (Fig. 4). Atropine had no effect on simultaneously recorded PBF in the unstimulated ipsilateral upper canine tooth, or during control measurements.

Cervical sympathetic stimulation

When the cervical sympathetic nerve was stimulated, a decrease in common carotid blood flow (CCBF) and PBF in the ipsilateral lower and upper canines was recorded in all experiments, as exemplified in Fig. 5. A moderate rise in systemic blood pressure was noted in some experiments. The fall in pulpal blood flow was partly blocked by phenoxybenzamine, whereas atropine had no effect (Fig. 5). In a total of 25 measurements (12 dogs), PBF fell on average to 35% of control (Fig. 2, middle panel), and calculated pulpal resistance rose by 50 to 1000%. Section of the sympathetic nerve caused no measurable change in PBF whereas CCBF rose on average 50%.

Effect of cutting the inferior alveolar nerve during cervical sympathetic stimulation

To test whether the pulpal vasoconstrictor fibers from the cervical sympathetic nerve run in the inferior alveolar nerve in the mandibular canal, the inferior alveolar nerve was cut

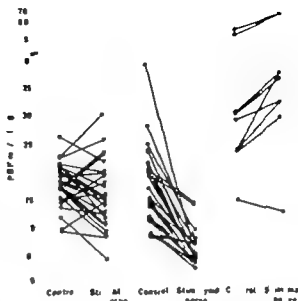


Fig 2 Pulpal blood flow (PBF) during electrical stimulation of the inferior alveolar nerve (left panel), the cervical sympathetic nerve (middle panel) and the mandibular nerve (right panel). Control measurements are indicated by open circles.

during cervical sympathetic stimulation. Typical responses are illustrated in Fig. 6. Nerve section had no effect on PBF in the ipsilateral upper canine tooth, whereas PBF in the lower canine abruptly increased in spite of continued sympathetic stimulation. Repeated sympathetic stimulation after cutting the inferior alveolar nerve gave a significant fall in PBF in upper canine, while PBF in lower canine was unchanged compared to control measurements (Fig. 6). Similar effects were obtained in 3 additional dogs. Systemic arterial pressure and CCBF were not affected by the denervation. As described above (Fig. 2), subsequent stimulation of the cut distal end of the inferior alveolar nerve had an inconsistent effect on PBF in the ipsilateral lower canine (Fig. 6).

The average fall in PBF during sympathetic stimulation before nerve section was 69% (4 dogs), whereas stimulation of the cut distal end of the inferior alveolar nerve caused a mean rise in PBF of 16% of controls in the same animals.

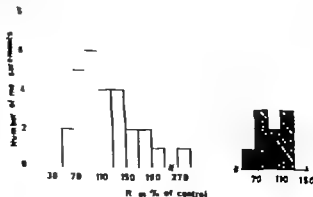


Fig 3 Pulpal vascular resistance (R) in lower canine tooth during afferent stimulation of the cut inferior alveolar nerve. Left panel: Before phenylephrine. Right panel: After phenylephrine, 7 mg/kg b.w.

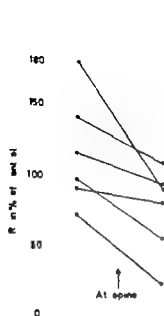


Fig. 4

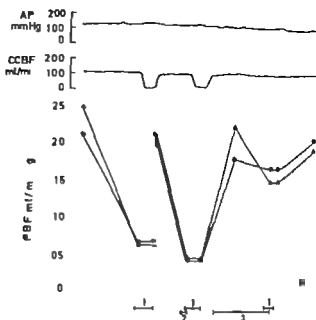


Fig. 5

Fig. 4. Pulpal vascular resistance (R) in per cent of control during inferior alveolar nerve stimulation (6 dogs). Open circles before, and closed circles, after atropine (0.3 mg/kg b.w.) administration.

Fig. 5. Simultaneous recordings of systemic arterial pressure (AP), common carotid blood flow (CCBF) and palpal blood flow (PBF) in ipsilateral upper (closed triangles) and lower (closed circles) canine tooth. 1. Cervical sympathetic stimulation 6 V 2 ms, 6 Hz, 2. I.v. infusion of atropine, 0.3 mg/kg b.w. 3. I.v. infusion of phenylephrine 50 μ g/min.

Mandibular nerve stimulation

The effect on PBF by efferent stimulation of the cut mandibular nerve before its entrance into the mandibular canal was studied in 5 dogs (9 measurements). During stimulation PBF in ipsilateral lower canine increased in all but one measurement (Fig. 2, right panel), on average by 27%. Systemic arterial pressure remained unchanged.

The majority of the present recordings during the various nerve stimulations were made with stimulus frequencies of 5 to 10 Hz. Frequency ranges of 1–5 or 10–20 Hz were used in only a few experiments. While these frequencies never revealed qualitatively different responses, the observations are too few to establish acceptable frequency/response curves.

Close intra-arterial infusion of noradrenalin

Close intra-arterial infusion of NA (0.01–5 μ g/min) always caused a fall in external carotid blood flow (ECBF) and PBF (8 dogs/70 measurements), as exemplified in Fig. 7. The vasoconstriction induced by NA infusion was only partly blocked by α -blockers (Fig. 7), and was never replaced by a vasodilator response (Fig. 8). In contrast, after α -blocking NA infusion increased ECBF by an average of 20% (SE 5%) indicating β -adrenergic vasodilator response in the nondental tissues supplied by the external carotid artery.

Close intra-arterial infusion of varying concentrations of NA was made for up to 16 min duration. Within this time interval there was no "escape" of the vasoconstrictor effect in the dental pulp, i.e. PBF remained low as long as the NA infusion was continued.

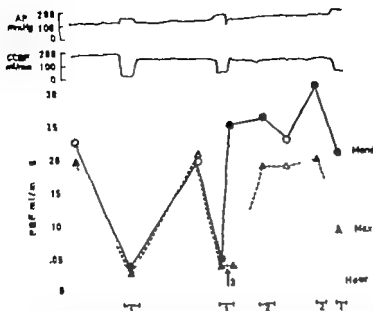


Fig. 6. Simultaneous recordings of systemic arterial pressure (AP), common carotid blood flow (CCBF) and palpal blood flow (PBF) in ipsilateral upper (triangles—broken lines) and lower (circles—solid lines), canine teeth. Open symbols represent the controls. 1. Cervical sympathetic stimulation 5 V 1 sec, 6 Hz. 2. Inferior alveolar nerve stimulation 5 V 1 sec, 6 Hz. 3. Section of the inferior alveolar nerve in the canal.

Usually the vasoconstriction lasted 1 to 4 min after NA infusion was stopped. Systemic arterial pressure rose slightly in some experiments. Mean pressure averaged 128 mmHg (SE 4 mmHg) in control periods and 132 mmHg (SE 3 mmHg) during catecholamine infusion. The vasoconstrictor response in the dental pulp was clearly dose dependent (Fig. 8). Doses of 5 $\mu\text{g}/\text{min}$ completely stopped H_2 -washout, while rates lower than 0.01 $\mu\text{g}/\text{min}$ gave no measurable change in PBF.



Fig. 7. Effect of dose (a. infusion of noradrenaline (11 $\mu\text{g}/\text{min}$) before and after 1. infusion of phenylephrine (50 $\mu\text{g}/\text{min}$, 15 min) on hydrogen desaturation curves from one electrode in the dental pulp. H_2 Hydrogen exhalation current (pneumographic plot). Simultaneous recordings of external carotid blood flow (ECBF) and systemic arterial pressure (AP).

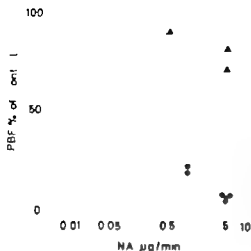


Fig. 8 Effect of various doses (0.01 to 5 µg/ml) of clonidine (clon.) on pupal blood flow (PBF). Closed circles, before and closed triangles, after phenoxybenzamine 7 mg/kg i.w.

Discussion

The experiments demonstrate clearly that the inferior alveolar nerve in the mandibular canal contains sympathetic α -adrenergic vasoconstrictor fibers (Fig. 6). In spite thereof, efferent stimulation of the cut inferior alveolar nerve produced variable pupal flow changes,—even pronounced vasodilation. Moreover, this vasodilation could be reinforced by intravenous infusion of atropine (Fig. 4). Thus, both increase and decrease in PBF were recorded during electrical stimulation of the inferior alveolar nerve, indicating that blood flow in the dental pulp in dogs may be influenced by both vasodilator and vasoconstrictor fibers.

Basal vasoactive nerve tone

Cutting of the cervical sympathetic nerve caused no change in PBF, suggesting nervous sympathetic basal tone in the pulp to be absent or low under the present experimental conditions. This conclusion agrees with the results reported by Edwall (1971) but is difficult to reconcile with the findings that section of the inferior alveolar nerve in the mandibular canal caused vasodilation (Fig. 1). Two possible mechanisms should be considered.

1. The inferior alveolar nerve contains tonically active vasoconstrictor fibers that are not severed by cutting the cervical sympathetic. Since α -adrenergic blockade did not enhance the dilatory effect of inferior alveolar nerve stimulation, this possible vasoconstrictor tone should not be mediated through α receptors.

2. Alternatively, the flow increase induced by inferior alveolar nerve section could be due to mechanical antidromic stimulation of trigeminal sensory fibers. The similar magnitude of the vasodilation caused by inferior alveolar nerve section and that caused by mandibular antidromic nerve stimulation would be compatible with this hypothesis. However, it should be emphasized that the results do not indicate a nervous vasodilator tone during normal conditions.

The possibility that removal of nervous vasoconstrictor tone could be compensated by autoregulation of pupal blood flow has been excluded by previous investigations (Tønder 1975).

Cervical sympathetic nerve stimulation and NA infusion

Electrical stimulation of the cervical sympathetic and NA infusion consistently increased pulpal vascular resistance. The constrictor effect was greatly reduced by α -blockers, but was never observed to be reversed to a vasodilator effect. Thus, the pulpal resistance vessels seem to be poorly equipped with β -receptors, supporting earlier observations where infusion of isoprenaline gave a weak if any pulpal vasodilation (Tønder 1976). Atropine, which should block a possible cholinergic vasodilation, had no effect on PBF during electrical stimulation of the cervical sympathetic. It seems therefore justified to conclude that the cervical sympathetic fibers have only one haemodynamic effect on the pulp, namely vasoconstriction, through activation of α -receptors.

Inferior alveolar nerve stimulation

The vasoconstriction elicited by cervical sympathetic stimulation was obviated by cutting the inferior alveolar nerve (Fig. 6), showing that the cervical sympathetic α -adrenergic fibers are contained in the inferior alveolar nerve. However contrary to expectation, stimulation of the inferior alveolar nerve did not consistently cause vasoconstriction (Fig. 2, left panel), and in several experiments a marked flow increase was observed. A similar variability in response was noticed by Weathered (1965), who reported both an increase and decrease in pulp pressure during inferior alveolar nerve stimulation. The variable flow response could theoretically be caused by varying activation of α and β -receptors by NA released at the sympathetic nerve endings. However this hypothesis is unlikely since 1. both responses were obtained with similar stimulation characteristics, and since 2. α -blockade (Fig. 5) and intra-arterial infusion of isoprenaline (Tønder 1976), gave little evidence for β -receptors. The inevitable conclusion therefore seems to be that the inferior alveolar nerve in addition to adrenergic fibers contains fibers which cause pulpal dilatation when activated. As will be discussed later the vasodilator response is most readily explained as an antidromic activation of sensory trigeminal fibers.

However this simple scheme fails to explain two rather surprising observations. In contrast to that observed during cervical sympathetic stimulation,—the constrictor response to inferior alveolar nerve stimulation was 1. reduced by atropine, but was 2. not appreciably influenced by phenoxybenzamine. The effect of atropine suggests that acetylcholine release plays a role in the constrictor response to inferior alveolar nerve stimulation. A constrictor effect of acetylcholine has previously been observed during intra-arterial infusion (Tønder 1976). The vasoconstriction might be due to a direct activation of vascular smooth muscle (Åström *et al.* 1964), or to enhanced release of catecholamines at sympathetic nerve endings (Ferry 1966). The latter mechanism seems unlikely since phenoxybenzamine had no effect on the response to inferior alveolar nerve stimulation. In fact, the lacking effect of atropine on the response to stimulation of the cervical sympathetic indicates that the cholinergic mechanism is not associated with sympathetic adrenergic fibers.

An effect of atropine on pulpal circulation has previously been reported by Weiss *et al.* (1972). They observed that electrical stimulation of the cut cervical sympathetic produced a decrease in "intrapulpal blood pressure" in the maxillary canine. This

response persisted in adrenergically depleted or α -adrenergic blocked dogs, but could be reduced or abolished by intravenous administration of atropine. Obviously these results are not in complete agreement with the present findings, but do suggest the presence of a cholinergic vasoactive mechanism in the dental pulp.

Mandibular nerve stimulation

Efferent stimulation of the mandibular nerve before its entry into the mandibular canal caused pulpal vasodilation (Fig. 2, right panel), which seems to indicate release of some vasodilatory substance at the sensory nerve endings. An axon reflex, like that involved in the trippel response in skin (Lewis 1927), would therefore seem possible also in the pulp, and might be of functional importance in the development of inflammatory vasodilation in the infected pulp. In fact the high control PBF measured after exposure of the mandibular nerve (Fig. 2, right panel), might suggest that the surgery itself had caused mechanical antidrome trigeminal stimulation and vasodilation. The prolonged vasodilator action of the unknown transmitter released in a sensory axon-reflex might thus explain why the increase in flow during mandibular nerve stimulation was so modest. We realize, however, that the present experiments are inadequate for a definite characterization of the vasodilator mechanism.

As described in an earlier study (Tonder 1976), vasodilation in neighbouring tissues may cause "stealing" of dental perfusion pressure, thus causing a reduction in PBF. During electrical nerve stimulation, not only the vascular resistance of the dental pulp was influenced, but also resistance in associated tissues. The prerequisite for this "steal" phenomenon is that there normally exists a considerable pressure gradient from the central arteries to the arterial ramification feeding the dental pulp. A pressure drop of on average 11 mmHg has previously been measured from the aorta to the lateral nasal artery (Tonder 1976), and the pressure drop to the small arterioles feeding the dental pulp may probably be of a greater magnitude. In the present study pressure in the arterioles directly feeding the dental pulp was not measured. However a simultaneous drop in pulpal perfusion pressure would mask a fall in pulpal vascular resistance during nerve stimulation. Accordingly pulpal vasodilation may have been underestimated.

To what extent blood flow changes in neighbouring tissues will affect PBF depends upon the normally existing ratio between intrapulpal to extrapulpal vascular resistance in the semicoupled vascular bed. Further research is therefore necessary to determine the normally existing pressure drop in the dental pulp. *I.e.* knowledge of the pressure in the small arterioles directly feeding, and the venules draining the dental pulp.

To summarize both increase and decrease in PBF was recorded during electrical nerve stimulation. Cervical sympathetic stimulation consistently caused a marked fall in PBF due to activation of α -receptors, while mandibular nerve stimulation before its entrance in the mandibular canal resulted in increased PBF in ipsilateral lower canine, probably due to the axonreflex mechanism. Efferent stimulation of the exposed inferior alveolar nerve in the mandibular canal gave variable PBF responses, presumably on account of that the inferior alveolar nerve is a mixed nerve, composed of both sensory adrenergic sympathetic and cholinergic fibers.

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Frequency selectivity of the peripheral auditory analyzer studied using broad band noise

By

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Abstract

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The sharpness of the frequency tuning of single auditory nerve fibers was studied in the rat on the basis of responses to broad band noise. The cross-spectra between the sound stimulus and the sound-to-stimulus locked discharge rate were used as estimates of the transfer functions of the peripheral auditory analyzer. The sharpness of the tuning obtained in that way was measured as Q_{max} and Q_{max} . It is found that these Q -values decreased with increasing stimulus intensity but that the decrease was different when it was based on measurements at 3 dB points of the obtained transfer functions compared with measuring it at 10 dB points. The change in width was less for fibers with low CF. In all the fibers studied the frequency of maximal response (CF) decreased with increasing sound intensity. The implication of these findings for pitch perception and for noise induced hearing loss is discussed.

Recent studies have provided much data on the frequency selectivity of the auditory periphery. A variety of different methods have been used to assess the frequency selectivity that can be measured in the cochlea, the basilar membrane and in single fibers of the auditory nerve (for reviews, see Möller 1972 and Evans 1975).

During the last two decades, measurements of the linear up-and-down motion of the basilar membrane have been performed in anesthetized animals by several investigators using different methods. The results of these measurements have been contradictory in many respects. Rhode (1971, 1973), Robles *et al.* (1976) using the Mössbauer effect, find that the motion of the basilar membrane is nonlinear within the physiological range of sound intensities. No other investigators have reported such findings. Johnstone and Boyle (1967) and Johnstone *et al.* (1969), using the same technique but in another animal species, find an essentially linear relationship between sound pressure and amplitude of motion. The results of other studies, in which a capacitive probe has served to measure the basilar membrane motion, also indicate that the basilar membrane function is a linear filter (Wilson and Johnstone 1972).

Several methods have been used to measure the frequency selectivity of the auditory

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periphery at the level of the auditory nerve. The most common method concerns the recording of frequency threshold curves (FTC) of single auditory nerve fibers in response to pure tones (see e.g. Kiang *et al.* 1965 and Evans 1975). Iso-intensity curves showing the discharge rate as a function of frequency for constant sound pressure have been determined (see e.g. Rose *et al.* 1971). A few studies have used isorate curves to show the sound pressure necessary to evoke a certain discharge rate (Gehrer *et al.* 1974). The results of these studies are difficult to compare because of the influence of the properties of the neural excitation process itself.

Determination of the response of single auditory nerve fibers to a short acoustic impulse (click) also gives information about the frequency selectivity properties of a frequency selective system. Such responses, however, are also influenced by the refractory properties of the neurons and do not reflect the frequency selective properties of the system only (see Kiang *et al.* 1965 Gray 1966). Recently stochastic signals have come into use in combination with recordings from single auditory nerve fibers and with statistical signal analysis for determining the filter characteristics of the auditory periphery (de Boer 1968 1969 Møller 1977 a, b, 1978, Evans 1977). This method makes it possible to estimate the frequency selective properties of the auditory periphery on the basis of the discharge pattern in single auditory nerve fibers over a large range of sound intensities (Møller 1977 a, b, Evans 1977). Because the stimulus is a continuous sound the influences of refractoriness on the response pattern is small compared with results obtained in response to stimulation with click sounds. In response to click stimulation the neuron will be refractory for a period following the first time it fires. That period of refractoriness may extend beyond the next excitatory phase of the basilar membrane motion. Using continuous stimulation diminishes these effects of refractoriness and consequently it is assumed that the present method makes it possible to estimate the frequency selective properties of the auditory periphery with a minimum of influence of the refractory properties (Møller 1975).

In previous work (Møller 1977 a, b, 1978), it was shown that the tuning of single auditory fibers in response to broad band noise changes in a systematic way such that the bandwidth of the estimated transfer functions increases with increasing sound intensity. In the present study the variation of bandwidth and center frequency as a function of sound intensity was studied for fibers with different center frequencies.

Methods

White rats weighing 200–300 g. are used. A hole is made in the occipital part of the skull and parts of the cerebellum were sucked away. Recordings were made with hypodermic glass pipettes filled with 2.5 mol potassium acetate and with resistance of 50–100 Mohms. The electrodes were placed in contact with the surface of the dorsal cochlear nucleus and aimed at the auditory nerve's entrance.

The electrode is advanced in 2 μ m steps by Salix microstimulator. The recorded potentials are amplified by capacitance compensated amplifier (Fomera). During the recording the animal was in a sound isolated box. The sound stimuli consisted of pseudorandom noise, generated by Hewlett Packard noise generator (type 3721A). The sound was fed into condenser microphones (Brüel & Kjær type 4131), which then served as the sound source, via conventional amplifiers and attenuators. The condenser microphone was connected to one of the hollow ear bars of the head holder. For further details about the recordings and sound generation, see Møller (1969, 1971). The sound generating system produced sound

Frequency selectivity of the peripheral auditory analyzer studied using broad band noise

By

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Abstract

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The sharpness of the frequency tuning of single auditory nerve fibers was studied in the rat on the basis of responses to broad band noise. The cross-spectra between the sound stimulus and the sound-to-stimulus locked discharge rate were used as estimates of the transfer functions of the peripheral auditory analyzer. The sharpness of the tuning obtained in that way was measured as Q_{max} and Q_{50} . It was found that these Q -values decreased with increasing stimulus intensity but that the decrease was different because it was based on measurements at 3 dB points of the obtained transfer functions compared with measuring it at 10 dB points. The change in width was less for fibers with low CF. In all the fibers studied the frequency of maximal response (CF) decreased with increasing sound intensity. The implication of these findings for pitch perception and for noise induced hearing loss is discussed.

Recent studies have provided much data on the frequency selectivity of the auditory periphery. A variety of different methods have been used to assess the frequency selectivity that can be measured in the cochlea, the basilar membrane and in single fibers of the auditory nerve (for reviews, see Möller 1972 and Evans 1975).

During the last two decades, measurements of the linear up-and-down motion of the basilar membrane have been performed in anesthetized animals by several investigators using different methods. The results of these measurements have been contradictory in many respects. Rhode (1971, 1973), Robles *et al.* (1976) using the Mössbauer effect, find that the motion of the basilar membrane is nonlinear within the physiological range of sound intensities. No other investigators have reported such findings. Johnstone and Boyle (1967) and Johnstone *et al.* (1969), using the same technique but in another animal species, find an essentially linear relationship between sound pressure and amplitude of motion. The results of other studies, in which a capacitive probe has served to measure the basilar membrane motion, also indicate that the basilar membrane function is a linear filter (Wilson and Johnstone 1972).

Several methods have been used to measure the frequency selectivity of the auditory

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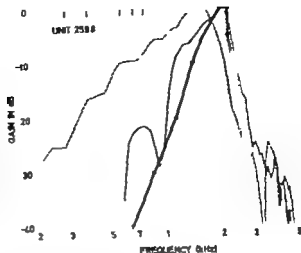


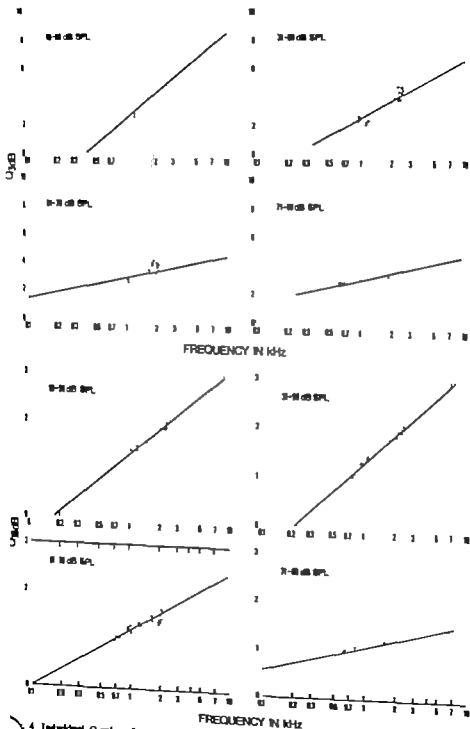
Fig. 2. Cross-spectra (transfer functions) obtained using noise stimuli 15 dB above threshold (thin continuous line) compared with the frequency threshold curve obtained using tone bursts (heavy solid line and dots). The dashed line is the transfer function obtained in response to noise stimuli at an intensity of 70 dB above threshold.

order to be classified as primary units, the units should not show injury potentials, contact with the units should be lost when the electrode was advanced a few microns and the shape of the recorded potentials should be monophasic.

Near threshold, the transfer function in response to noise stimuli of many of the units resembles the frequency threshold curves (FTC) obtained with conventional methods employing tone bursts. Fig. 2 shows a comparison between the frequency curve (heavy line) and the transfer function computed on the basis of noise stimulation at a level near threshold (thin solid line). It is seen that there is a fairly good agreement between the frequency threshold curve (FTC) and the transfer function computed for noise whose level is just above threshold. The dashed line shows the transfer function computed in the same way but for an intensity of 55 dB above that shown by the thin solid line. The transfer function obtained at a 55 dB higher noise level (dashed line) has a broader peak and the curve is shifted downwards in frequency. This widening of the peak and shift downward in frequency are typical findings.

The change in center frequency (CF) and in width of the transfer functions for two typical fibers is further illustrated in Fig. 3 A and B. The peak value is normalized and the various curves shifted 5 dB vertically in order to facilitate comparison. These two units have different CF's and it is seen that the CF shifts downwards as the intensity is increased. The shift in CF is, however, less for the unit with low CF than for the one with a higher CF.

The Q-value (CF value divided by the width of the obtained transfer functions) was higher for fibers with high CF than for those with low. At low sound levels the Q-value increases more rapidly with CF than at high sound levels. This is illustrated in Fig. 4 where the Q_{3dB} and Q_{10dB} (CF divided by the width of the transfer functions measured at 3 resp. 10 dB below the peak value) are shown for the fibers studied. These two figures show individual data points for the individual fibers at the different sound intensities plotted as a function of the fiber's CF with the linear regression lines superimposed. The sound intensities are indicated by legend numbers. The spread of the data is considerable and the linear regression lines may not adequately describe the experimental data.



4 Individual Q-values for the fiber studied plotted as a function of CF. Each data point is the intensity range indicated in each of the four graphs. The straight lines are linear

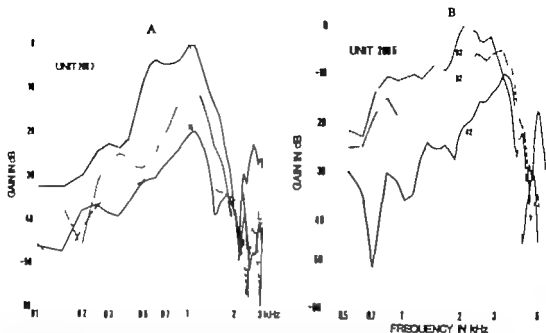


Fig. 3 Frequency transfer functions obtained in two different fibers. The individual curves were obtained at different sound levels, indicated by legend numbers, in dB SPL measured in a $\frac{1}{2}$ octave band. The curves are shifted vertically in such a way that there is a 5 dB difference between the maximal values of the individual curves. This was done in order to facilitate comparison of the width and center frequency of the individual curves.

The change in CF as a function of sound intensity is shown in Fig. 5 A for units with CF below 1 400 Hz and in Fig. 5 B for units with CF above 1 400 Hz. It is seen that the CF of all the units decreases with increasing sound level, but that the spread between units is much greater for the units with CF below 1 400 Hz than for those with CF above 1 400. For units with CF above 1 400 Hz a systematic and gradual decrease in CF with intensity is seen. The decrease in CF is most pronounced for intensities above 40 dB SPL. At 80 dB SPL the CF has decreased to between 70 and 80% of the value it had at intensities near threshold. The data given in Fig. 5 concern units on which results were obtained over a large range of intensities and thus does not represent the total material.

Discussion

In the present study the change in the width and CF of the computed transfer function (cross-spectra) of primary auditory fibers in response to broad band noise as a function of the intensity of the noise was studied. The change in the width of the transfer functions of primary fibers was found to reveal a different course depending on whether the width of the derived transfer functions was measured at 10 dB points or 3 dB points. The CF decreases with increasing intensity in all the units studied.

Frequency threshold curves (FTC) measured in the cat by Kiang *et al* (1965) showed higher Q_{10dB} values than those obtained in the present study at noise levels just above threshold. Kiang's data showed a mean Q_{10dB} value of about 3 for fibers with CF at 1 kHz compared with 1.3 in the present study for intensities near threshold. At 3 kHz, the

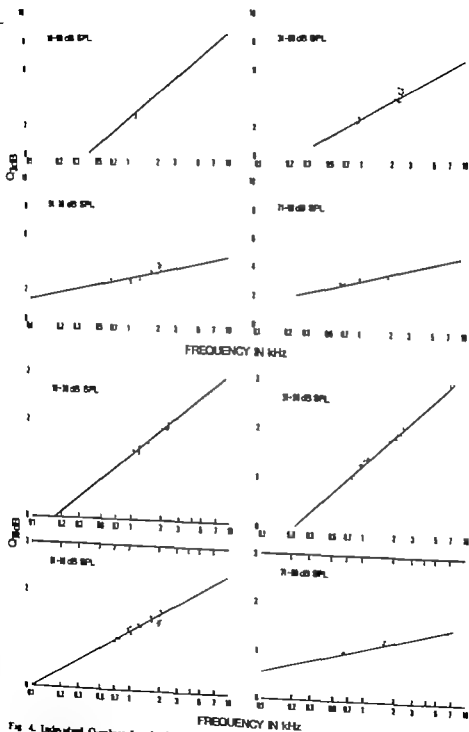


Fig. 4. Individual Q -values for the fiber studied plotted as a function of CF. Each data point is the Q -value obtained in the frequency ranges indicated in each of the four graphs. The straight lines are linear regression lines.

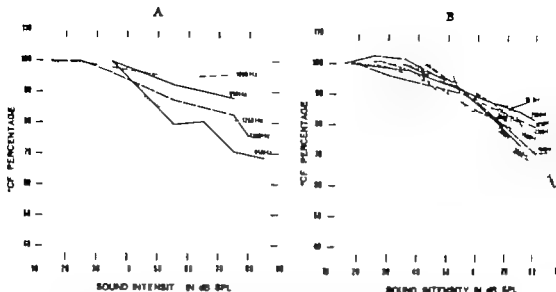


Fig. 5 Change in CF as a function of sound level (given in dB SPL in $\frac{1}{2}$ octave band) for a number of typical units. The ordinate is CF in percentage of its value at intensities near threshold. The CF of the units is given by legend numbers. A Units with CF below 1400 Hz, B Units with CF above 1400 Hz (736)

Q_{10dB} for the FTC's obtained in the cat is about 6 whereas the Q_{10dB} obtained in the present study at 10–30 dB SPL is about 2.3. At 4 kHz, the mean value of the Q_{10dB} of the FTC's is greater than 10. The Q_{10dB} determined on the basis of the responses to noise stimulation was about 2.5 near threshold for fibers with CF around 4 kHz. The above given Q_{10dB} of FTC in the cat (Kiang *et al.* 1965) agrees well with the Q_{10dB} in the guinea pig reported by Evans and Wilson (1973).

It is thus obvious from these results that the FTC's obtained using pure tones are narrower (at 10 dB points) than the transfer functions obtained in the present study using noise stimuli. Despite this general rule, the FTC and transfer functions in response to noise in an individual fiber may resemble each other (cf Fig. 2) as has been shown by de Boer (1969) as well.

It is also of interest to compare the frequency tuning of the basilar membrane shown by Rhode (1971, 1973) in the squirrel monkey with the results of the present study. He found a tuning at 8 kHz and 70 dB SPL that had a Q_{10dB} of about 4. The present study does not include fibers with CF as high as 8 kHz but extrapolation suggests that Q -values for such CF's would be less than 4 at 70 dB SPL.

It is seen from Fig. 3 that the change in bandwidth with increasing sound intensity is mainly a result of a decrease of the low frequency slope of the transfer functions. The high frequency slope is almost unchanged. The slope of the low frequency part of the transfer functions is to a great extent a result of the transmission characteristics of the middle ear. The absolute value of the bandwidth consequently becomes greatly dependent on transmission properties of the middle ear. The characteristics of the middle ear however do not change with sound intensity. Thus, although the bandwidth measurement may not be a good measure of frequency selectivity, the change in bandwidth with sound intensity may represent a true measure of the change in the frequency selectivity of the peripheral analyzer with sound intensity.

The shift in CF downwards in frequency with increasing sound intensity seen in the present study implies that the maximal deflection of the basilar membrane, when produced by a pure tone, shifts towards the base of the cochlea. Indeed, psychoacoustic experiments show no dependence of pitch perception on intensity (Terhardt 1975) (except for pure tones where there is a slight change in pitch with intensity). The change in CF observed in the present study on the other hand may explain why noise induced hearing loss caused by noise in the form of pure tones or narrow band noise as well as the temporary threshold shift after exposure to such sounds is greatest at a frequency about one half to one octave above the frequency of exposure. A hearing-damaging sound with a narrow band (as e.g. a pure tone or narrow band noise) will hence damage the hair cells at a point of the basilar membrane which, at threshold intensities, responds to a frequency that is higher than the frequency of the sound in question.

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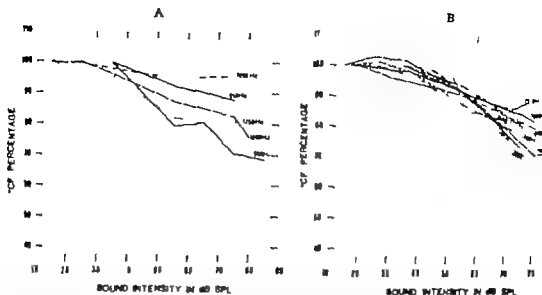


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Efferent innervation of the small intestine by adrenergic neurons from the cervical sympathetic and stellate ganglia, studied by retrograde transport of peroxidase

By

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Abstract

LUNDBERG, J. M., A. DAHLSTRÖM, I. LARSSON, G. PETTERSSON, H. AHLMAN and J. KEWENTER.

Efferent innervation of the small intestine by adrenergic neurons from the cervical sympathetic and stellate ganglia, studied by retrograde transport of peroxidase Acta physiol. scand. 1978. 104. 33-42.

The seroton pathways between the small intestine of cat and guinea pig and various sympathetic ganglia were investigated by the retrograde horseradish peroxidase (HRP) technique. HRP was injected at multiple sites in the wall of the duodenum and the first third of the jejunum. At 1-5 days after the injections, the HRP reaction product was searched for in various sympathetic ganglia. Not only the coeliac and nodose ganglia, but also the superior cervical, stellate and thoracic ganglia contained HRP-positive nerve cells. Crushing the cervical vagal nerve prevented the occurrence of HRP-reaction in the cervical ganglia, indicating that the HRP was transported from the gut to the cervical ganglia via axons in the vagal nerve. The results demonstrate that the sympathetic ganglia in the neck (sup. and med. cerv. ganglia and stellate ppl.) send efferent fibres to the small intestine.

En vänd strömning duodenum, HRP injektion, retrograd transport, adrenerge perikarya, cervical sympatiska ganglia, vagus nerv

Several observations indicate that the serotonin (5-HT) content in enterochromaffin cells in the small intestine of mammals may be influenced by stimulation of efferent nerve fibres in the cervical vagus (Höbenkötner *et al.* 1971 Tansy *et al.* 1971 Ahlman *et al.* 1976 a). If adrenergic β -receptor-blocking agents were administered prior to nerve stimulation, or if the superior cervical ganglion (SCG) were removed 1 week before the stimulation experiment, the release of 5-HT from enterochromaffin cells was abolished (Ahlman *et al.* 1976 b). This indicated that an adrenergic pathway running in the cervical vagus was causing the 5-HT release by liberating a catecholamine (CA). By nerve crush experiments it has been shown that the cervical vagal nerve contains descending CA-containing fibres, probably originating in the SCG (Muryobayashi *et al.* 1968, Liedberg

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Efferent innervation of the small intestine by adrenergic neurons from the cervical sympathetic and stellate ganglia, studied by retrograde transport of peroxidase

By

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The nerve pathways between the small intestine of rat and guinea pig and various sympathetic ganglia were investigated by the retrograde horseradish peroxidase (HRP) technique. HRP was injected at multiple sites in the wall of the duodenum and the first third of the jejunum. At 1-5 days after the injections, the HRP reaction product was searched for in various sympathetic ganglia. Not only the coeliac and mesenteric ganglia, but also the superior cervical, medial cervical, stellate and thoracic ganglia contained HRP positive nerve cells. Crushing the cervical vagal nerve prevented the occurrence of HRP-reaction in the cervical ganglia, indicating that the HRP was transported from the gut to the cervical ganglia via axons in the vagal nerve. The results demonstrate that the sympathetic ganglia in the neck (sup. and med. cerv. ganglia and stellate ggl.) send efferent fibres to the small intestine.

Key words: mesenteric duodenos, HRP injection, retrograde transport, adrenergic perikarya, cervical sympathetic ganglia, vagus nerve

Several observations indicate that the serotonin (5-HT) content in enterochromaffin cells in the small intestine of mammals may be influenced by stimulation of efferent nerve fibres in the cervical vagus (Hohenstein *et al.* 1971, Tanay *et al.* 1971, Ahlman *et al.* 1976a). If adrenergic β -receptor-blocking agents were administered prior to nerve stimulation, or if the superior cervical ganglia (SCG) were removed 1 week before the stimulation experiment, the release of 5-HT from enterochromaffin cells was abolished (Ahlman *et al.* 1976b). This indicated that an adrenergic pathway running in the cervical vagus was causing the 5-HT release by liberating a catecholamine (CA). By nerve crush experiments it has been shown that the cervical vagal nerve contains descending CA-containing fibres, probably originating in the SCG (Muroybayashi *et al.* 1968, Liedberg

TABLE I The presence of HRP reaction product in neuronal structures of the guinea pig after local injections of HRP in the wall of the small intestine. HRP-injections and nerve crush operation performed 24 h before death. + Presence of HRP-positive cells or fibres. 0 = no HRP product observed. -- = not studied.

Structure	Crush operation			
	- (n=3)	cerv.-vagus unilat. (n=2)	cerv. vagus+ cerv. preagl. nerve unilat. (n=2)	cerv. vagus bilat. (n=2)
Cerv. vagus				
ipsilat.	-	+	+	+
contralat.	-	-	-	+
Cerv. preagl. nerve				
ipsilat.	-	0	0	-
contralat.	-	-	-	-
Coeliac ggl.	+	+	+	+
Stellate ggl.				
ipsilat.	+	+	+	+
contralat.	+	+	+	+
Sup. cerv. ggl.				
ipsilat.	+	0	0	0
contralat.	+	+	+	0

et al 1973, Ahlman *et al* 1976 b). However, the direct connection between these fibres and the small intestine was never established. The purpose of the present investigation was therefore to perform experiments which could demonstrate a direct morphological pathway between the small intestine and the SCG.

Exogenous proteins are known to be taken up by nerve terminals of various kinds of neurons and transported via retrograde intra-axonal transport towards the cell body. A protein which has been frequently used for studies of retrograde intra-axonal transport and for tracing nervous pathways is horse-radish peroxidase (HRP) (*cf.* Kristensson and Olsson 1971, Ellison and Clark 1975). This protein was therefore used in our studies and injected into the small intestine and traced, by histochemical procedures, in various ganglia and the vagal nerve.

Material and methods

Injections of HRP: 9 adult male guinea pigs and 7 adult cats of both sexes were used. HRP (Type II, Sigma) dissolved in saline (>50%), was injected at multiple sites into the wall of the duodenum and the proximal $\frac{1}{2}$ of the jejunum. The injections were made under aseptic conditions: laparotomy under Nembutal (cats, 35 mg/kg i.p.) or ether (guinea pigs) anaesthesia. The total volume of HRP-solution injected into each animal was 250 (μ l) (guinea pig) or 500 (μ l) (cat), and each injection consisted of 1-3 μ l of the solution (freshly prepared for each animal). The survival time was between 1-5 days (*see* Table I and II). The postoperative care of the cats consisted of heating pads or lamps during the recovery after anaesthesia, and fluid administration if necessary (indicated by weight loss) during the following days. Water and food was supplied *ad libitum*.

Nerve operations: In 6 of the 9 guinea pigs the cervical vagal nerve alone or together with the adjacent cervical sympathetic preganglionic nerve was crushed, uni- or bilaterally (Table I). In 3 cats the right cervical vagal nerve, together with the adjacent cervical preganglionic nerve were crushed 0 or 24 h (Table II) after the HRP injections. The crushes were performed *s.m.* Lubidzka (1959) with silk sutures.

TABLE II. The presence of HRP-reaction product in neuronal structures of the CAT after local injections of HRP in the wall of the small intestine. Nerv. operations performed as indicated. + = HRP positive structures present, 0 = no HRP reaction product observed — = not investigated.

Cat No.	I	II	III	IV	V	VI	VII
Time [cat] — outlet nerve op.	0 h	24 h	24 h	—	—	0 h	—
Time nerve op. — death	24 h	36 h	48 h	—	—	3 d (subdiaph. vag. cat bilat.)	—
Total survival time:	24 h	60 h	3 days	2 days	3 days	3 days	5 days
Structures							
Cerv. vagus:							
spinal	0	+	+				
coeliac		—	—				
Cerv. preggl. nerve:							
spinal	0	0	0				
coeliac	—	—	—				+
Coeliac ppl.	+	+	+	+	+	+	
Stellate ppl.							
spinal	0	+	+	+	+	0	+
coeliac	0	+	—	+	+	0	+
Med. cerv. ppl.							
spinal	—	+	+	—	—	—	—
coeliac	—	+	+	—	—	—	—
Sup. cerv. ppl.							
spinal	0	0	0	0	+	0	+
coeliac	0	+	+	0	+	0	+
Meduse ppl.							
spinal	0	0	0	0	+	0	—
coeliac	0	+	+	0	+	0	+
Vagus thoracic ppl.		—	—	—	—	—	+

(3) II under the nerve(s) back was pulled firmly (3–5 s) against glass rod placed over and along the nerve(s). This produced straight and very clear interruption of the nerve(s) but with least consecutive tissue damage. 1 one cat (Table II) both subdiaphragmatic nerves were traced and cut at the time of the HRP injections.

The histochemical demonstration of HRP. At the end of the survival period, guinea pigs were reanesthetized with ether and cats with Nembutal. Following brief pre-perfusion (30 s) with Ringer-Rheonacrodex (Pharmacia) the animals were then perfusion-fixed with ice-cold 2.5% glutaraldehyde in 0.15 M phosphate or Tris-buffer, pH 7.4. After dissection of appropriate tissues these were postfixed for 2 h (guinea pigs) or 5 h (cats) in the same fixative at +4°C. The specimens are rinsed in buffer with 5% sucrose overnight and then sectioned at about 30 μ m (ganglia) or 10 μ m (nerves) in cryostat at -18–20°C. The sections are then incubated with 3,3'-diamino benzidine (DAB) followed by 0.01% hydrogen peroxide according to Grisham and Karnovsky (1966) or with the modifications described by Malmgren and Olsson (1977). The sections were then mounted in 50% glycerine and stored at -20°C until examined in phase contrast microscope. The DAB-labelling procedure causes dark brownish precipitate over areas with HRP-activity. Granules with HRP-activity were in most cases clearly different from the yellowish lipofuscin pigment present in many cells. In doubtful cases, however the reaction as studied in fluorescence microscope, in which lipofuscin granules are strongly yellowish autofluorescent while the HRP-reaction product is dark. Kodak Tri-X film was used for photographs.

Dissected tissues were: The coeliac ganglion (mesenteric inferior ganglion), the stellate, nodal cervical (MCG), superior cervical (SCG) and nodose ganglia bilaterally and the thoracic chain ganglia (cat 7 Table II). When nerve crush operations were performed the cranial end of the nerves caudal to the crush were dissected out.

TABLE I The presence of HRP reaction product in neuronal structures of the guinea pig after local injections of HRP in the wall of the small intestine. HRP-injections and nerve crush operation performed 24 h before death. + Presence of HRP-positive cells or fibres, 0 = no HRP product observed, — = not studied.

Structure	Crush operation			
	— (n = 3)	cerv. vagus unilat. (n = 2)	cerv. vagus + cerv. pregangl. nerve unilat. (n = 2)	cerv. vagus bilat. (n = 2)
Cerv. vagus				
ipsilat.	—	+	+	+
contralat.	—	—	—	+
Cerv. pregangl. nerve				
ipsilat.	—	0	0	—
contralat.	—	—	—	—
Coeliac gangl.	+	+	+	+
Stellate gangl.				
ipsilat.	+	+	+	+
contralat.	+	+	+	+
Sup. cerv. gangl.				
ipsilat.	+	0	0	0
contralat.	+	+	+	0

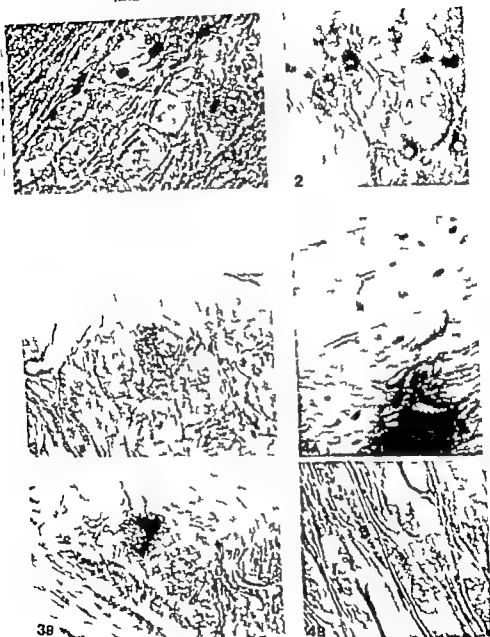
et al. 1973, Ahlman *et al.* 1976 b). However the direct connection between these fibres and the small intestine was never established. The purpose of the present investigation was therefore to perform experiments which could demonstrate a direct morphological pathway between the small intestine and the SCG.

Exogenous proteins are known to be taken up by nerve terminals of various kinds of neurons and transported *via* retrograde intra-axonal transport towards the cell body. A protein which has been frequently used for studies of retrograde intra-axonal transport and for tracing nervous pathways is horseradish peroxidase (HRP) (*cf.* Kristensson and Olsson 1971, Ellison and Clark 1975). This protein was therefore used in our studies and injected into the small intestine and traced, by histochemical procedures, in various ganglia and the vagal nerve.

Material and methods

Injections of HRP. 9 adult male guinea pigs and 7 adult cats of both sexes were used. HRP (Type II, Sigma) dissolved in saline (> 50%), was injected at multiple sites into the wall of the duodenum and the proximal $\frac{1}{2}$ of the jejunum. The injections were made under aseptic conditions (laparotomy under Nembutal (cats, 35 mg/kg i.p.) or ether (guinea pigs) anaesthesia. The total volume of HRP-solution injected into each animal was 250 (guinea pig) or 500 (cat) μ l, and each injection consisted of 1–3 μ l of the solution (freshly prepared for each animal). The survival time was between 1–5 days (see Table I and II). The postoperative care of the cats consisted of heating pads or lamps during the recovery after anaesthesia, and fluid administration if necessary (indicated by weight loss) during the following days. Water and food was supplied *ad libitum*.

Nerve operations. In 6 of the 9 guinea pigs the cervical vagal nerve alone or together with the adjacent cervical sympathetic preganglionic nerve was crushed, uni- or bilaterally (Table I). In 3 cats the right cervical vagal nerve together with the adjacent cervical preganglionic nerve were crushed 0 or 24 h (Table II) after the HRP injections. The crushes were performed *a.m.* Labliska (1959) with a silk suture



Figs. 1-4

caudal to the crush (Fig. 5). In the cervical sympathetic trunk no accumulation of HRP product was demonstrated in relation to the crush (see Table I and II). These experiments also show that in cat the retrograde transport of HRP had not reached the level of the crush at 24 h.

Results

In both species the injection of HRP into the small intestine caused dark reaction product to appear after the DAP-procedure in cell bodies of all ganglia investigated after a sufficient transport time, *i.e.* celliac, stellate, nodose ganglia, MCG and SCG (Table I and II). There was a positive reaction in cell bodies of all these ganglia in guinea pigs already 4 h after injection (Fig. 1). In cats only the celliac ggl. had HRP positive cells 24 h after injection (Fig. 2). If the observation time of cats was 60 h (cat II, Table II), relatively few cell bodies of the cervical ganglia contralateral to the nerve crushes contained HRP-reaction product, and the amount of product in the positive cells was rather small (Fig. 3). After 7th h, however cats III-V VII (Table II), the number of positive cells had increased, and more material seemed to have accumulated in the cells (Fig. 4-5). In the cervical sympathetic ganglia of the 3 days surviving cats the HRP reaction product was distributed fairly evenly in the few positive cells observed. In the 5 days cat, however (cat VII, Table II), the HRP reaction product was often accumulated near one or both poles of the nucleus, thus resembling the location of lipofuscin pigment in these neurons (Figs. 4-5).

By studying these cells in the fluorescence microscope it was demonstrated, however, that these granules were not yellowish autofluorescent as is lipofuscin. They rather appeared dark in the fluorescence microscope (Fig. 6).

The thoracic ganglia also contained a few HRP positive cells, but the amount of HRP in these cells was usually rather small (Fig. 7).

Crush operation of the cervical vagal nerve uni- or bilaterally 0 or 24 h after the HRP-injection prevented in both guinea pigs and cats the occurrence of HRP reaction product in the supraclavicular ganglia of the ipsilateral side: thus the nodose ganglia, the SCG and the MCG contained no visible HRP-positive cells. The ganglia on the contralateral side of unilaterally operated animals had positive cells (Table I and II). The infraclavicular ganglia, *i.e.* the stellate and the coeliac ganglia, had the same appearance as in the unoperated animals. The crush-operated animals were also used as a control that no blood-borne HRP was causing the positive results in the supraclavicular ganglia. The vagal nerve of these crush-operated animals contained many axons with accumulated HRP-reaction product.

Fig. 1. Nodose ganglion of a HRP-injected but not nerve-crushed operated guinea pig, containing one HRP-positive (arrows) and several negative nerve cells. BC indicate red blood cells with endogenous peroxidase activity. $\times 270$.

Fig. 2. Coeliac ganglion from a cat injected with HRP 3 days earlier (cat III, Table II). Many HRP positive cells, with various amounts of HRP-granules in the cytoplasm, can be seen. $\times 70$.

Fig. 3. A, B. From the stellate ganglion of a cat injected with HRP in the small intestine 60 h (cat II, Table II) before death. Single HRP-positive cells are seen red amongst many negative cell bodies. Few (A) or many (B) HRP granules are distributed rather evenly in the cytoplasm. $\times 270$.

Fig. 4. Stellate ganglion of a cat injected with HRP in the small intestine 3 d. before death. A) Many cell bodies with HRP-reaction product are observed. Note the irregular distribution of HRP to one or two poles of the nucleus. $\times 65$ B) Three HRP positive cells with the HRP-granules located near one pole of the nucleus. These granules were not fluorescent in the fluorescence microscope and are thus not lipofuscin pigment. $\times 270$.

Discussion

The HRP-method for studying retrograde transport in axons, and thereby tracing nervous pathways, has been used for some years with excellent results (cf. Kristensson 1975). In all types of neurons investigated so far including peripheral autonomic neurons, HRP is taken up into the nerve endings and transported by intra-axonal retrograde transport to the perikarya (cf. Kristensson and Olsson 1971, 1973; LaVail and LaVail 1972; Ellison and Clark 1975). The rate of transport towards the cell bodies is rapid, probably exceeding 120 mm/day in the cat and guinea-pig (Ellison and Clark 1975). This figure is in good agreement with our observations that in the cat no HRP-positive cell bodies in the neck ganglia were labelled at 24 h after the HRP-injection, while at 60 h (cat 2 in Table II) some cells were labelled with numerous granules of HRP-reaction product. In the guinea-pig, however, strongly positive cells were observed at all levels 24 h after the HRP-injection. The distance between the duodenum and the cervical ganglia was estimated to be at least 30 cm in the cat and about 10 cm in the guinea-pig, indicating a rate of transport of more than 100-120 mm/day for both species.

Autonomic nerves are considered to be particularly suitable for HRP-studies due to their large surface/volume relationship (Nauta *et al.*, 1974). Also, the more terminals belonging to particular perikaryon that are present in the HRP-injected area, the more HRP is taken up and transported to the cell body (Jones 1975). Thus, the amount of HRP-granules present in a cell body can be related to its amount of nerve terminals in the injected area. This may explain why at a certain survival time a particular ganglion may contain cell bodies which are filled with HRP-granules close to other cell bodies with only a few HRP granules or no granules at all. This was observed in all ganglia investigated, but particularly evident in the celiac ganglion (Fig. 2).

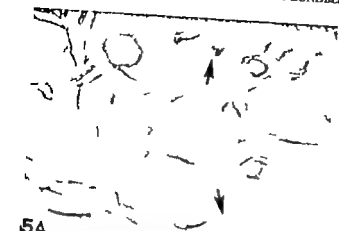
In this study all ganglia investigated after a sufficient transport time contained HRP granules after injection of this exogenous protein into the small intestine. This is unlikely to be due to uptake of blood-borne HRP for two reasons. 1) In animals with nerve crushes

Fig. 5 Cervical superior ganglion of a cat injected with HRP in the small intestine 3 d. before death (cat VII in Table II). A) T cells (arrows) with rather small amount of HRP-reaction product in the cytoplasm are seen. 65 B) Two HRP-positive cells with few HRP-granules, located near one pole of the nuclei can be observed (arrows). These granules were non-fluorescent in the fluorescence microscope and are therefore not lipofuscin granules. 290.

Fig. 6 Set of photographs of stellate ganglion of cat VII in Table II. A) Phase contrast picture demonstrating dark HRP-reaction product in one cell (arrow). A few dark red blood cells are also seen (→). B) Fluorescence microscope picture of the same area as in A. The HRP-reaction product in the nerve cell appears dark (arrow), and the same cells with the red blood cells, also containing the DAB-peroxidase reaction product (→). 85.

Fig. 7 Ganglion Th 5 in the thoracic sympathetic chain of cat injected with HRP in the small intestine 5 days before death (cat VII in Table II). A) Two HRP-positive nerve cell bodies (arrow heads) and several negative cells (arrows) are observed. 65. B) One nerve cell with HRP-granules accumulated in the cytoplasm in one pole of the cell. These granules are non-fluorescent and thus probably not lipofuscin granules. 290.

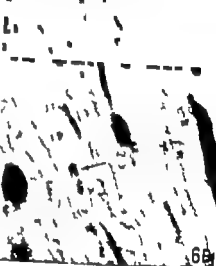
Fig. 8 The distal end of the cervical vagal nerve of cat II (Table II). 30 h after the HRP injection into the small intestine the vagal nerve is crushed (site of crush indicated by arrow head). The animal was killed 14 h later. Dark brown granular HRP reaction product can be seen in axonal structures caudal to the crush (arrows). 290.



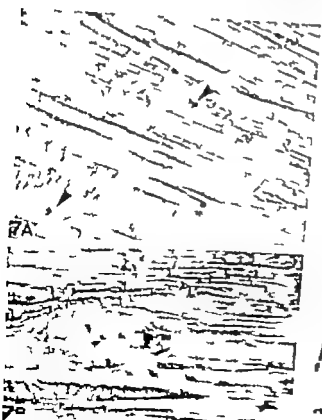
5A



5B



6B



7A



ganglia, probably rise the vagal nerve. The evidence for this is that when the vagal nerve, with or without intact cervical preganglionic trunk, was crushed, HRP was found in accumulated fibres distal to the crush but not in the cervical preganglionic nerve. Crushing of the cervical preganglionic trunk alone could not prevent the appearance of HRP in the SCG (Table I), nor were HRP-accumulations observed in this nerve in either cats or guinea-pigs. It is therefore probable that some of the HRP-containing nerve fibres in the cervical vagus may correspond to the CA-1 fibres in the vagal nerve which originate in the SCG (These fibres disappear after removal of the SCG). However the vagal nerve fibres with HRP-accumulations may also correspond either to afferent fibres, constituting the major part of the vagal nerve (Agostoni *et al.* 1957), to efferent parasympathetic nerve fibres, or to polypeptide containing nerve fibres in the vagal nerve (Lundberg *et al.* unpublished).

The cervical sympathetic ganglia of cat, and probably also of guinea pig, contain not only adrenergic perikarya but also a few non-fluorescent, acetylcholine-esterase (AChE)-positive cells which probably are cholinergic (*cf.* Hamberger *et al.* 1965). These cells are not possible to recognize unless specific AChE-staining is performed. So far no attempt has been made to identify the HRP-positive cells in the present study as adrenergic or cholinergic nerve cells. However 1) cholinergic neurons of the cervical and stellate ganglia of cat are considered to innervate eccrine sweat glands (*cf.* Dale and Feldberg 1934, Sjogqvist 1963), and 2) the subdiaphragmatic vagal nerve of cat contains many descending adrenergic fibres emanating from the cervical and stellate ganglia (Lundberg *et al.* 1973, Ahlman *et al.* 1976 b). Therefore it is probable that the HRP-positive perikarya are adrenergic in nature rather than cholinergic.

The function of this presumably adrenergic fibre system from the SCG and MCG to the intestine is so far not clear. However it may participate in the vagal adrenergic release of 5-HT from enterochromaffin cells, which is caused by selective efferent stimulation of the cervical vagal nerve (Ahlman *et al.* 1976 b) or in the vagally induced inhibitory motor responses from the small intestine (Martin *et al.* 1974).

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Two of the HRP-positive ganglia in this study are known to send nerve endings to the small intestine. Thus, the concept of the *nodose ganglion* is that it contains parasympathetic afferent neurons, which innervate the splanchnic area with autonomic sensory fibres via the vagal nerve. In agreement with this view Ellison and Clark (1975) observed many HRP-positive ganglion cells in this ganglion following the injection of HRP into the small intestine of cats and guinea pigs. This was prevented by crushing the vagal nerve before or just after the HRP injection. Our present results confirm the results reported by Ellison and Clark in all details.

The *coeliac ganglion* is well known to send postganglionic adrenergic fibres to the stomach and the small intestine (cf Furness and Costa 1974). The most likely anatomical pathway for this innervation and for the HRP-transport is the postganglionic fibres which *via* blood vessels reach the gut.

It has been suggested earlier that the *stellate ganglion* mainly supplying the heart and cervical-thoracic structures with CA-fibres, also sends CA-fibres to the small intestine (Liedberg *et al* 1973). The number of HRP-positive cells in this ganglion was rather great, especially in the 5 days cat. The HRP is likely to have reached the stellate ganglia via the vagal nerve and communicating branches between the stellate and other intrathoracic ganglia and the thoracic portion of the vagus. This is supported by the observation that many CA-fibres, presumably originating mainly from the stellate ganglia, are present in the vagus at the subdiaphragmatic level after removal of the SCG (Liedberg *et al* 1973; Ahlman *et al* 1976 b; see also Ahlman and Larsson 1978).

The SCG and MCG were classically considered to innervate adrenergically the structures of the head and neck. The present study demonstrates that some cell bodies in this ganglion also supply CA-fibres to the small intestine, which is a most surprising observation (Fig. 6). Earlier methods for tracing nervous adrenergic pathways were rather insensitive compared to the HRP-method. Such methods included on the one hand, nerve crush experiments which demonstrated the presence of adrenergic fibres in a peripheral nerve and also gave information on the direction in which the fibres were running. On the other hand, denervation experiments were performed in which a particular ganglion or set of ganglia was removed about 1 week before fluorescence histochemical or quantitative investigations on a possible decrease in CA-nerve terminals in a certain area (cf Hamberger and Norberg 1964). With the use of the HRP method, which may demonstrate even a very small relative innervation, it is possible to study the autonomic innervation to the gastrointestinal tract in much greater detail than earlier.

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The relationship between cortical recruiting responses and ponto-geniculo-occipital waves during paradoxical sleep in the cat

By

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Abstract

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In an earlier study it was found that during paradoxical sleep (PS) thalamo-cortical recruiting responses (RRs) and rapid eye movements usually did not appear simultaneously. As shown elsewhere, ponto-geniculo-occipital (PGO) activity and rapid eye movements are during PS closely related to each other in time. Similarly, in the present study it is observed that during paradoxical sleep in cats in which the central median nucleus of the thalamus was being stimulated at a rate of 7-9 Hz PGO waves in the geniculate nuclei did not in the rule occur in the presence of RRs recorded from the motor cortex. This effect was most pronounced with respect to series of PGO waves which usually occur at rates of 4-7 Hz. On the basis of these experiments it is concluded that PGO waves and RRs are reciprocal events and mutually exclusive. Considering the well-known fact that RRs represent synchronization, this negative correlation between RRs and PGO waves indicates that the desynchronizing tendency typical to paradoxical sleep is most pronounced during the occurrence of PGO waves.

Key words: Paradoxical sleep, ponto-geniculo-occipital waves, recruiting responses.

Since Dempsey's and Morrison's original discovery (1942 a, b) concerning the recruiting response (RR) which is seen in the cortex during the repetitive stimulation of the non-specific thalamic nuclei, there have been numerous studies in attempt to clarify this thalamo-cortical relationship and especially its functional significance at various levels of vigilance. Though Dempsey and Morrison in their original publication in 1942 studied RRs only in cats under barbiturate anesthesia, it was later shown that RRs could be elicited also during slow wave sleep (S) (Evarts and Magoun 1957, Roelke *et al.* 1961) and—if the thalamic stimulation was slightly suprathreshold compared to that during S—even during certain parts of paradoxical sleep (PS) and quietly alert state (Evarts and Magoun 1957, Yamaguchi *et al.* 1963, Yamaguchi *et al.* 1964). Earlier (Allison 1965, Lehtinen and Valleala 1969) it was shown that RRs and rapid eye movements during PS did not usually occur simul-

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The relationship between cortical recruiting responses and ponto-geniculo-occipital waves during paradoxical sleep in the cat

By

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Abstract

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In an earlier study it was found that during paradoxical sleep (PS) thalamo-cortical recruiting responses (RRs) and rapid eye movements usually did not appear simultaneously. As shown elsewhere, ponto-geniculo-occipital (PGO) wave activity and rapid eye movements are during PS closely related to each other in time. Similarly as the present study it was observed that during paradoxical sleep in cats in which the caudal median nucleus of the thalamus was being stimulated at rates of 7-9 Hz PGO waves in the geniculate nuclei did not in the rule occur in the presence of RRs recorded from the motor cortex. This effect was most pronounced. As respect to series of PGO waves which usually occur at rates of 4-7 Hz. On the basis of these experiments it was concluded that PGO waves and RRs are reciprocal events and mutually exclusive. Considering the well-known fact that RRs represent synchronization, this negative correlation between RRs and PGO waves indicates that the desynchronizing tendency typical to paradoxical sleep is most pronounced during the occurrence of PGO waves.

Key words: Paradoxical sleep, ponto-geniculo-occipital waves, recruiting responses.

Since Dempsey's and Morrison's original discovery (1942a, b) concerning the recruiting response (RR) which is seen in the cortex during the repetitive stimulation of the non-specific thalamic nuclei, there have been numerous studies in attempt to clarify this thalamo-cortical relationship and especially its functional significance at various levels of vigilance. Though Dempsey and Morrison in their original publication in 1942 studied RRs only in cats under barbiturate anesthesia, it was later shown that RRs could be elicited also during slow wave sleep (S) (Evarts and Magoun 1957, Rossi *et al.* 1961) and—if the thalamic stimulation was slightly suprathreshold compared to that during S—even during certain parts of paradoxical sleep (PS) and quietly alert state (Evarts and Magoun 1957, Yamaguchi *et al.* 1963, Yamaguchi *et al.* 1964). Earlier (Allison 1963, Lehtinen and Valleala 1969) it was shown that RRs and rapid eye movements during PS did not usually occur simul-

taneously. In the present study it was found that ponto-geniculo-occipital (PGO) waves did not either usually occur in the presence of RRs during PS. A preliminary report on the present study has been published (Laihinen and Valleala 1977).

Methods

The experiments were performed on 4 adult unanesthetized chronic cats carrying teflon-coated stainless steel electrodes previously implanted under barbitalurate anesthesia. During the experiments the cats were sleeping on a hammock with the head firmly fixed to a frame by a dental cement pedestal built on the skull. Stimulating electrodes consisted of two wires, each 0.25 mm in diameter. They were insulated at their obliquely cut ends, cemented side by side and their tips were separated vertically by 0.5 mm. The stimulating electrodes were inserted in the left center median nucleus according to Jasper and Ajmonia-Mariani's atlas (1954).

The stimuli consisted of rectangular pulses of 0.5 ms duration at a rate of 7-9 Hz. Stimulation was provided by a Grass S-4 stimulator and an RF isolation unit. The stimulus strength needed to elicit RRs during PS was slightly suprathreshold compared to that which had to be used during slow sleep. The recruiting responses were recorded from the ipsilateral anterior sigmoid cortex by using a transcortical electrode set and were identified by their long latency (about 20 ms), surface negative incremental growth and amplitude modulation of waxing and waning character. The PGO waves were detected with bipolar electrodes in the lateral geniculate nuclei.

After experimental series which could last several weeks the positions of the stimulating electrodes and those in the geniculate nuclei were marked by electrolytic deposits of iron. The points were then checked histologically in serial sections which were stained with cresyl violet (Nissl).

Results

In Fig. 1 there is a sample of PS with a recruiting response which was elicited by means of the repetitive stimulation of the center median nucleus and recorded from the anterior sigmoid cortex. At the beginning of the sample the stimulus strength (7 V) is not sufficient to evoke RRs. After a slight increase in the stimulus strength (to 8 V) the recording shows with every pulse a long latency (about 20 ms) potential. At the very beginning of the repetitive stimulation these potentials do not exhibit any systematic amplitude modulation. These potentials have been called sustained waves (Wells and Sutlin 1964). However in the middle of the sample, a recruiting-like amplitude modulation can be seen. There is an essential difference between the classical RR and the recruiting phenomenon described in Fig. 1.

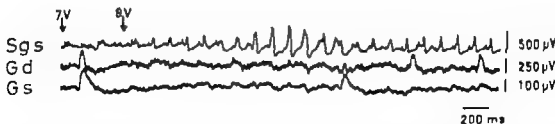


Fig. 1. Electrical activity of the left anterior sigmoid cortex (Sgs) and the right (Gd) and left (Gs) lateral geniculate nuclei. The cortical recording shows recruiting response (RR) elicited by repetitive stimulation (9 Hz, 0.5 ms) of the left center median nucleus. After the increase in the stimulus strength from 7 V to 8 V every stimulus evokes in the left anterior sigmoid cortex long-latency surface negative wave ("sustained wave") which in the course of stimulation shows a recruiting-like amplitude modulation. During RR there are no ponto-geniculo-occipital (PGO) waves in the tracing from the right and left lateral geniculate nuclei. Upward deflection signifies here and in Fig. 2 surface negativity.

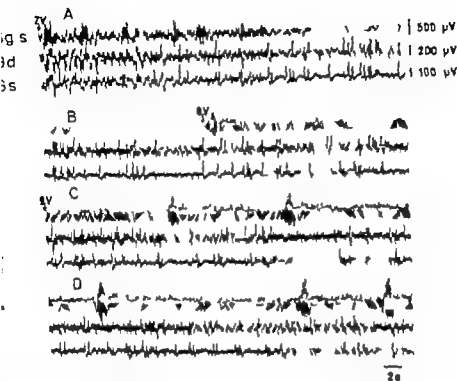


Fig. 2. Continuous recording during the transition from S to PS (tracing A) and PS proper (tracings B-D). Thalamic stimulation (9 Hz, 0.5 ms) is going on the whole time. The stimulus strength, which is at the threshold level for the occurrence of RRs during S, had to be raised to a higher level in order to evoke correct RRs during PS. Sg s., G d., and O.s. as in Fig. 1. PGO wave activity of G d. and O.s. does not occur during RRs but may start immediately after the end of each RR (e.g., tracing D).

12. that in the classical case the incremental change begins from the zero level whereas in his atypical case during PS the incremental rise is initiated from the level of the sustained waves. There are no PGO waves in the lateral geniculate nuclei during the RR (Fig. 1).

Fig. 2 shows 4 tracings (A-D) representing the same continuous recording during which the repetitive thalamic stimulation is going on the whole time. In the tracing A, the transition from S to PS is shown. It is noteworthy that S changes to PS in spite of the simultaneous thalamic stimulation. In this tracing there are RRs evoked at the threshold stimulation during S. The last few RRs during S overlap the first PGO waves heralding PS, but do not occur strictly simultaneously with these waves. The tracings B-D in Fig. 2 represent the fully developed PS. At the beginning of the tracing B before increasing the stimulus strength, neither sustained waves nor RRs are seen. After an increase in stimulus strength (in the middle of tracing B) there appears an initial RR and sustained wave activity but not until a further increase in stimulation voltage (at the beginning of tracing C) do the recruiting responses become recurrent. In the presence of RRs PGO waves are absent not only in the left geniculate nucleus but also contralaterally in the respective nucleus.

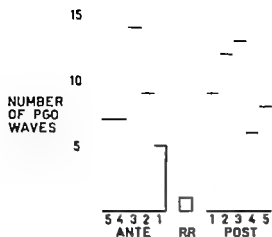


Fig. 3 Number of PGO waves in both the lateral geniculate nuclei during a PS episode of 3 min length. The repetitive stimulation of the left center median nucleus was going on the whole PS episode eliciting totally ten RRs, the average length of which amounted to 1.3 s. PGO waves were sampled during these RRs and in five 1 s periods before (ANTE) and after (POST) RRs. For details, see text.

Fig. 3 shows the number of PGO waves in both the lateral geniculate nuclei during a PS episode of 3 min length. The repetitive stimulation of the center median nucleus was going on the whole PS episode eliciting totally ten RRs, the average length of which amounted 1.3 s. The time immediately preceding and following every RR of the sample was divided into five 1 s periods and the total number of PGO waves occurring in both lateral geniculate nuclei was determined separately for each period and during RRs. Fig. 3 shows that on average once did a PGO wave occur during RR. The typical series of PGO waves at a rate of 4 Hz never occurred during RRs.

Discussion

The results of the present study point out that PGO waves and RRs are aspects of opposite physiological tendencies. Generally PGO waves refer to an increase and RRs to a decrease in the level of activation. The electrocortical desynchronization and the PGO wave activity are among the main bioelectric characteristics of PS (Jouvet 1972). On the contrary the conventional RRs have been characterized as signs of the electrocortical synchronization. It seems that also the modified type of RRs encountered during paradoxical sleep as described in the earlier (Lehtinen and Valleala 1969) and present studies is of the same quality. It is obvious, though not yet experimentally verified, that PGO wave activity picked up from the pontine reticular formation has the same type of relationship with RRs. In fact, that region is one of likely sites of interaction of these two factors. That type of interaction could be possible provided that the effects of the repetitive thalamic stimulation can reach the critical brain stem region, which could happen in two principal ways, directly (Schlag and Faidherbe 1961) or indirectly e.g. via the cortical motor area and the respective descending tracts (Lehtinen 1972). The possibility that the lateral geniculate nuclei have been influenced by intrathalamic connexions cannot be excluded. However, it seems unlikely because of the fact that the scarcity of PGO waves during RRs is clearly noticeable not only in the ipsilateral but also in the contralateral hemisphere with respect to the site of the thalamic stimulation. The most likely explanation is that the desynchronizing tendency of the PGO wave mechanism is the primary factor influencing the non-specific thalamocortical projection system, and thus preventing the appearance of RRs.

Repetitive stimulation of the non-specific thalamus can have behavioral effects like a cessation of the on-going motor activity in an alert animal (Hunter and Jasper 1949 Rouguel *et al* 1967). Correspondingly during PS, there are no rapid eye movements in the presence of RRs elicited by such a thalamic stimulation. In the present study it was shown that also central cerebral mechanism—in this case PGO wave activity which is closely connected with the phasic motor phenomena typical to PS—can interact with RRs.

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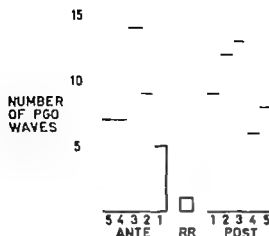


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1977 b) This was produced by progressive coronary artery stenosis and was accompanied by a 20% decrease in diastolic coronary flow, an unchanged systolic flow and a relative endocardial hypoperfusion. The distribution of coronary blood flow was estimated from the activity concentrations of Xe-133 in tissue blocks after bolus injection of the isotope into the aortic root. The reduced diastolic coronary blood flow produced by the stenosis might facilitate diffusion of xenon from vessels supplying the endocardial region during their passage through the epicardial part of the wall. This would result in activity concentrations which would not be proportionate to flows. Downey *et al.* (1975) have suggested that when perfusion pressure is reduced below a critical level stress relaxation of the subendocardial vasculature occur too slowly for adequate subendocardial perfusion. This would result in a lower endo- than epicardial blood flow reserve but only at decreased perfusion pressures.

In order to avoid substantial reductions of total coronary blood flow and perfusion pressure coronary vasodilatation was produced in a group of dogs by progressive hemodilution, until the diastolic reactive hyperemic response to 10 s occlusion of the left circumflex coronary artery just disappeared. In accordance with reports concerning experimental coronary artery stenosis (Reneman and Spencer 1972, Van Der Meer 1972, Bagger 1977 b) coronary vasodilatation was considered at a maximum when the diastolic reactive hyperemic response was abolished. At this degree of hemodilution ("optimum hemodilution") the distribution of coronary blood flow in the left ventricular wall was estimated by measuring tissue activity concentrations of Xe 133 and radioactive microspheres injected as a bolus into the aortic root.

Occlusion of a coronary artery for 10 s results in a maximum vasodilatation peripheral to the occlusion (Olsson and Gregg 1965, Khouri, Gregg and Lowensohn 1968, Van Der Meer 1977). Furthermore, the occlusion leads to depression of myocardial contractility and systolic intramyocardial pressure (Van Der Meer 1972). In order to evaluate the influence of these changes on the distribution of coronary blood flow the left coronary artery was occluded for 10 s in another group of dogs. In this group myocardial activity concentrations were measured after injection of Xe 133 and radioactive microspheres at peak diastolic reactive hyperemia.

Methods

20 mongrel dogs of both sexes (24-49 kg) were anesthetized with pentobarbital sodium 25 mg kg⁻¹ i.v. Ventilation was maintained with atmosphere air (0.275 l kg⁻¹ min⁻¹) through an endotracheal tube by means of Servotrachistor-900 (Siemens-Elema).

Local anesthetic (Lidocaine-mesopropylfumarate 1%, 5 ml) was injected into both inguinal regions and the left and right femoral arteries and right femoral vein were exposed. Dacor® "pig-tail" and left coronary artery catheters were introduced through the left and right femoral arteries respectively. Under fluoroscopy the tip of the "pig-tail" catheter was positioned in the left ventricle for measurements of diastolic pressure. The tip of the left coronary artery catheter was positioned in the aortic root without pointing at the region of the left coronary artery. This catheter was used for measurements of aortic pressure and injection of tracers. ECG was recorded in standard limb lead I or II. After injection of local anesthetic into the thoracic all positive and expiratory pressures of 5 cm of water was established to prevent collapse of the lungs. Thoracotomy was performed on the left side and the pericardium was opened. The bifurcation of the left coronary artery was exposed by blunt dissection. A close-fitting electromagnetic flow transducer was placed on the circumflex branch and connected to square-wave flowmeter (Nycotron type 371-3 or 376). ECG, aortic pressure and left ventricular diastolic pressure were recorded continuously on an oscilloscope and for two periods, immediately after positioning the catheters and after exposing the left coronary ar-

Distribution of maximum coronary blood flow in the left ventricular wall of anesthetized dogs

By

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Abstract

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Blood flow in the circumflex branch of the left coronary artery was recorded by electromagnetic flow meters. In a group of dogs progressive hemodilution was performed until the diastolic reactive hyperemic response to 10 s occlusion of the circumflex branch disappeared ("optimum hemodilution"). At this degree of hemodilution the distribution of blood flow in the left ventricular free wall was evaluated by measuring tissue activity concentrations of Xe-133 and radioactive microspheres after bolus injection into the aortic root. Optimum hemodilution was accompanied by a sixfold increase in systolic coronary flow, a 3-fold increase in diastolic flow and a relative endocardial hypoperfusion. These results indicate that the endocardial blood flow reserve is lower than the epicardial. This conclusion is supported by the influence of spontaneous oscillations of arterial blood pressure (Traube-Hering waves) on systolic and diastolic coronary blood flows before and during "optimum hemodilution". In another group of dogs maximum coronary vasodilatation was produced by occlusion of the left coronary artery for 10 s. In this group the distribution of Xe-133 and radioactive microspheres were measured after bolus injection into the aortic root at peak diastolic reactive hyperemia. The epi- and endocardial distribution of both Xe-133 and microspheres was uniform in the left ventricular wall indicating uniform flow in these regions. This might be explained by an increased endocardial perfusion during systole due to loss of myocardial contractility or by a decline towards resting level in epicardial flow at the time of injection, corresponding to a shorter duration of the hyperemic period in the epi- than endocardial region.

Myocardial uptake of different diffusible tracers have indicated that blood flow per unit weight is uniform in the left ventricular wall of normal anesthetized dogs (Moir and DeBris 1967, Griggs and Nakamura 1968, Bagger 1972). This has been confirmed in some experiments with radioactive microspheres of less than 15 μm in diameter (e.g. Becker, Fortuin and Pitt 1971). Downey and Kirk (1974) have shown that systolic coronary flow mainly supplies the epicardial part of the left ventricular wall. Therefore, uniform flow indicates that diastolic flow is larger and diastolic vascular resistance smaller in the endo- than epicardial region. As a consequence the blood flow reserve might be lower endo- than epicardially.

The relative epi- and endocardial blood flow reserves have previously been evaluated by the distribution of coronary blood flow at maximum coronary vasodilatation (Bagger

TABLE 1. Hemodynamic and respiratory parameters (mean \pm SD) in the groups of dogs subjected to hemodilution or reactive hyperemia. c: control, d: after isolation of the coronary arteries, h: at optimum hemodilution or peak reactive hyperemia. LVDP: left ventricular diastolic pressure. Hgb: hemoglobin concentration. Ht: hematocrit.

Aortic pressure mmHg		Heart rate min ⁻¹	LVDP mmHg	Hgb g 100 ml ⁻¹	Ht	P O ₂ mmHg	P CO ₂ mmHg	pH
Systolic	Diastolic							
<i>Optimum hemodilution and occlusion of the left anterior descending branch (group D₂) (N = 6)</i>								
c	129 ± 35	98 ± 28	83 ± 29	6 ± 4		95 ± 9	26 ± 3	7.40 ± 0.04
d	134 ± 39	112 ± 34	138 ± 29	3 ± 4	13.3 ± 1.3	44 ± 5	97 ± 7	27 ± 4
h	128 ± 33	91 ± 24	146 ± 26	6 ± 6	3.3 ± 0.6	11 ± 2	106 ± 9	29 ± 4
<i>Optimum hemodilution only (group D₃) (N = 4)</i>								
c	128 ± 18	104 ± 20	84 ± 33	9 ± 3	12.2 ± 1.9	39 ± 5	92 ± 9	28 ± 2
d	144 ± 18	121 ± 16	138 ± 28	7 ± 5				
h	136 ± 23	104 ± 26	160 ± 21	9 ± 2	4.1 ± 0.4	14 ± 2	97 ± 15	31 ± 3
<i>Peak reactive hyperemia (group D₄) (N = 9 unless otherwise stated)</i>								
c	127 ± 14	98 ± 15	92 ± 23	8 ± 2			90 ± 9	31 ± 6
d	140 ± 20	116 ± 17	180 ± 30	3 ± 4	13.8 ± 1.1	45 ± 4	97 ± 4	32 ± 4
					N = 4	N = 4	N = 4	N = 4
h	129 ± 25	101 ± 24	171 ± 35	6 ± 4			94 ± 8	31 ± 7
				N = 8			N = 8	N = 8

mean values of measurements performed immediately after introduction of the catheters (c) after isolation of the left coronary artery (d) and at optimum hemodilution or peak reactive hyperemia (h). "Optimum hemodilution was accompanied by decreases in hemoglobin concentration and hematocrit to 1/3-1/4 of the initial values. In group D₄ no changes are observed in the hemodynamic parameters after occlusion of the left anterior descending branch.

Fig. 1 shows the ECG, left ventricular diastolic and aortic pressures and the instantaneous flow in the left circumflex branch before, during and after 10 s occlusion of the branch. The recordings are before the start of hemodilution (A) at a dilution which reduced the diastolic R-H-response (B) and at optimum hemodilution (C). The figure illustrates that optimum hemodilution was accompanied by a relatively larger increase in minimum flow during systole ("systolic flow") than in maximum flow during diastole ("diastolic flow"). For the optimum hemodiluted dogs of group D "systolic" flow increased by factor 5.7 (range 3.7-8.0), while "diastolic" flow increased by a factor 3.3 (range 2.1-4.6). The figures are significantly different ($p < 0.0001$ by method of paired comparison).

Fig. 2 shows the distribution of microspheres in one dog before hemodilution (control) and of microspheres and Xe 133 in the same dog at optimum hemodilution and occlusion of the left anterior descending branch. The slices are numbered in the direction from the apical region towards the base of the heart. The activity concentrations of the tissue blocks of each slice are plotted in the direction from the area supplied by the left anterior descending branch towards that supplied by the circumflex branch. Before hemodilution the distribution of microspheres is uniform. At optimum hemodilution the levels of activities are very low

tery also on a 4-channel ink jet recorder (Mingograf 34 Elema Schonander). After application of the flow transducer ECG pressures and the instantaneous flow in the left circumflex branch were recorded continuously on this recorder.

11 dogs (group D) were submitted to hemodilution. In this group a snare for 10 s occlusions was placed distal to the flow transducer. The left carotid artery was exposed and a Bardoc catheter was introduced. The tip of a polyethylene catheter was positioned in the inferior caval vein via the right femoral vein. Progressive hemodilution was performed replacing blood by 10% dextran (mol. wt. 40,000) in 0.9% saline (Rheomacrodex®) during frequent recordings of the reactive hyperemic response (RH-response) to 10 s occlusion of the circumflex branch. Initially 10–25 ml of blood per kg body weight were drawn from the carotid artery and replaced by the same amount of dextran solution infused into the inferior caval vein. With increasing coronary blood flow and decreasing RH response the infused volume of dextran was reduced to 40–100 ml between each recording of the RH response. In 10 dogs hemodilution was continued until the diastolic RH-response just disappeared ("optimum" hemodilution). This was obtained in 33–278 min. In 6 of these dogs (group D) the anterior descending branch of the left coronary artery was occluded at its origin, Xe-133 (0.63 mCi in 2.4 ml saline) (one dog) or a mixture (vol. 3.0–5.5 ml) of Xe-133 (1.4–4.0 mCi) and radioactive microspheres (13–22 μ Cl Ce-141) (5 dogs) was immediately injected as a bolus into the aortic root and the heart excised at once. The time interval between start of injection and disappearance of the ECG was 4–7 s. Control injection of microspheres (39–69 μ Cl Cr 51 in 5 ml 10% dextran) into the aortic root was performed prior to hemodilution in the same 5 dogs. In other 4 dogs (group D) submitted to optimum hemodilution occlusion of the left anterior descending branch was omitted. Xe-133 dissolved in saline (0.63–1.96 mCi in 2.4–2.9 ml) was injected as a bolus into the aortic root and the heart excised at once. In this group 4–6 s elapsed between start of injection and disappearance of the ECG. In the last dog of group D hemodilution was continued beyond the disappearance of the diastolic RH-response until an increasing left ventricular diastolic pressure was observed. Then Xe-133 (0.84 mCi in 2.4 ml) was injected as a bolus into the aortic root and the heart excised at once. The time interval between start of injection and disappearance of the ECG was 6 s.

9 dogs (group R) without hemodilution were used for measurements of flow distribution during peak diastolic reactive hyperemia. A loose ligature was placed around the main stem of the left coronary artery for occlusion. Xe-133 (0.63–1.96 mCi in 1.4–3.4 ml) (5 dogs) or a mixture (vol. 2.9–5.0 ml) of Xe-133 (0.7–1.66 mCi) and radioactive microspheres (11–13 μ Cl Ce-141) (4 dogs) was injected as a bolus into the aortic root at peak diastolic coronary flow after 10 s occlusion of the left coronary artery and the heart excised at once. The time interval between start of injection and disappearance of the ECG was 3–14 s.

After introduction of the catheters, before hemodilution and immediately before injection of tracer oxygen and carbon dioxide tensions and the pH of arterial blood were measured. In all dogs submitted to hemodilution and in 4 dogs of group R additional measurements of hematocrit and hemoglobin concentration were performed.

Polystyrene microspheres (3M Company) labelled with Cr 51 or Ce-141 with diameter of 9 ± 1 (μ m) and a density of 1.23 g cm^{-3} were used for the experiments. They were obtained as 1 mCi of nuclide in 10 ml 10% dextran containing 0.05% polyoxyethylene 80-sorbitan mono-oleate (Tween-80) to minimize clumping. The specific activities were about 35 mCi g⁻¹ and 10 mCi g⁻¹ for spheres labelled with Cr 51 and Ce-141 respectively. The number of microspheres per injection was $6 \cdot 10^6$ – $9 \cdot 10^6$. No changes in the hemodynamic parameters were observed after the injections.

After excision of the heart the previously described procedure was followed (Bagger 1972). In brief the heart was frozen in a mixture of dry ice and iso-pentane (-75°C). The apex was removed and the heart was sliced at right angles to the long axis. From each slice the left ventricular free wall was divided into blocks. Each block was subdivided further into septal and endocardial halves and placed in previously weighed, cooled, closed tubes. The activities were counted in a well-type scintillation detector with the windows of its two channels adjusted around the 320 keV peak of Cr 51 and the 81 keV peak of Xe-133. The tubes were weighed again and the caps were removed for 24 h to allow xenon to escape. The activities were then counted with the windows adjusted around the 320 keV peak of Cr 51 and the 145 keV peak of Ce-141. The activities of each isotope were calculated and expressed as cps 100 mg⁻¹ or cpm 100 mg⁻¹.

Results

Table I shows the hemodynamic and respiratory parameters measured in the ten dogs of group D subjected to "optimum" hemodilution and in all dogs of group R. The figures are

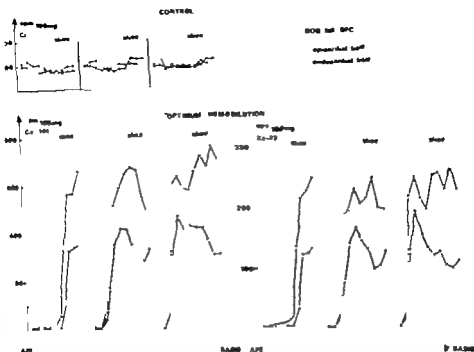


Fig. 2. The distribution of microspheres in epi- and endocardial halves of 3 slices of the left ventricle of one dog before hemodilution (control) and of microspheres and Xe-133 in the same slices at "optimum" hemodilution and occlusion of the left anterior descending branch. The activity concentrations of the tissue blocks of each slice are plotted in the direction from the anterior descending (A.D.B.) towards the circumflex supplied area.

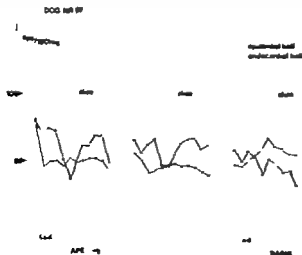


Fig. 3. The distribution of Xe-133 in the left ventricular wall of one dog plotted as in Fig. 2. Xenon was injected at "optimum" hemodilution and unimpeded flow in the left anterior descending branch.

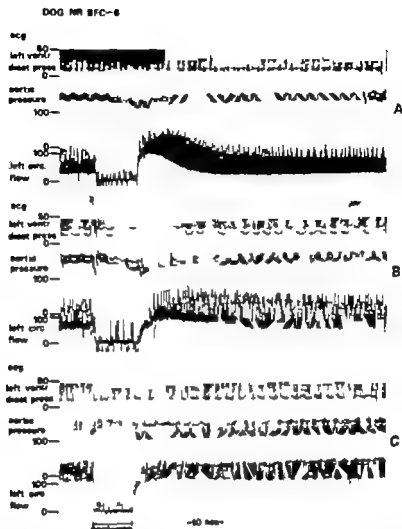


Fig. 1 ECG left ventricular diastolic and aortic pressures (mmHg) and the instantaneous flow in the left circumflex branch (arbitrary units) before during and after occlusion for 10 s. A before hemodilution. B at a dilution which reduced diastolic reactive hyperemic response C at optimum hemodilution.

in the area supplied by the occluded left anterior descending branch. In the region supplied by the circumflex branch the levels of activities are significantly higher and furthermore, the activities of both microspheres and Xe 133 are substantially higher in the epi- than endocardial region. Table II shows the mean ratio of epi- to endocardial activity concentrations of microspheres before hemodilution and of microspheres and Xe 133 at "optimum" hemodilution and occlusion of the left anterior descending branch. The ratio of microsphere activities in the circumflex area is significantly higher at "optimum" hemodilution than the corresponding control value ($p < 0.01$ by method of paired comparison). The ratio of microsphere activities and Xe 133 are not significantly different at "optimum" hemodilution ($p > 0.05$ by method of paired comparison).

Fig. 3 shows the distribution of Xe-133 in three slices of the left ventricular wall of an "optimum" hemodiluted dog with unimpeded flow in the left anterior descending branch. The activity concentrations are plotted as in Fig. 2 and demonstrate a higher epi- than

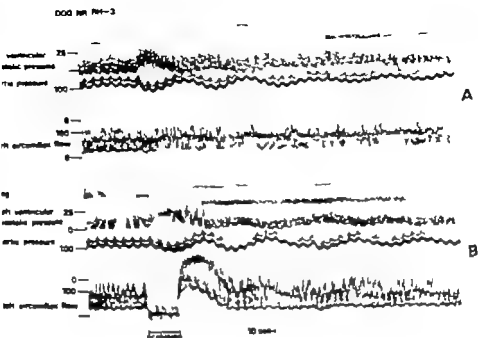


Fig. 3 Trache-Henry waves generated spontaneously (A) and during 10 occlusions of the left coronary artery (B).

paired comparison). Compared to the ratio obtained in hemodiluted dogs with occluded left anterior descending branch (1.81 ± 0.11) the corresponding ratio is significantly lower ($p < 0.05$).

The last dog of group B was hemodiluted beyond the disappearance of the diastolic RH-response and until signs of left ventricular insufficiency appeared. Left ventricular diastolic pressure increased from 3 (before the onset of hemodilution) to 47 mmHg, aortic systolic and diastolic pressures decreased from 161 to 106 mmHg and from 136 to 86 mmHg respectively. Diastolic coronary flow was reduced to control level, while "systolic" flow was twice as high as the initial value. Fig. 4 shows the distribution of activity in the left ventricular free wall of this dog plotted as in Fig. 2. The ratio of epi- to endocardial activities is 3.11 ± 0.15 (SE), which is significantly higher than in the circumflex supplied area of "optimum" hemodiluted dogs with occlusion of the left anterior descending branch ($p < 0.01$).

In group R "systolic" and "diastolic" flows were increased by factors 4.6 (range 3.2-7.0) and 2.6 (range 1.8-4.0) respectively at peak reactive hyperemia. These factors are significantly different ($p < 0.005$ by method of paired comparison). Table II shows the mean ratios of epi- to endocardial activity concentrations in this group. The ratio for Xe 133 is significantly higher in the circumflex than in the left anterior descending area ($p < 0.05$ by method of paired comparison). Compared to the ratio in the circumflex area of hemodiluted dogs with unimpeded flow in the left anterior descending branch (1.38 ± 0.06) the corresponding ratio in group R is significantly lower ($p < 0.02$). The ratio of microsphere ac

TABLE II Ratios of epi- to endocardial activity concentrations of Xe-133 and radioactive microspheres in the left ventricular free wall (mean \pm SE). L.a.d. and circumflex areas regions supplied by the left anterior descending and circumflex branches respectively

	L.a.d. area	Circumflex area	Whole left ventricular wall
<i>Control (group D₁).</i>			
microspheres (N=5)	0.86 ± 0.05	0.92 ± 0.05	0.88 ± 0.05
<i>"Optimum" hemodilution and occlusion of the left anterior descending branch (group D₂).</i>			
microspheres (N=5)		1.72 ± 0.14	
Xe-133 (N=6)		1.81 ± 0.11	
<i>"Optimum" hemodilution only (group D₃)</i>			
Xe-133 (N=4)	0.98 ± 0.07	1.38 ± 0.06	1.18 ± 0.07
<i>Peak react. hyperemia (group H).</i>			
microspheres (N=4)	0.85 ± 0.16	0.90 ± 0.10	0.88 ± 0.11
Xe-133 (N=9)	0.90 ± 0.06	1.05 ± 0.07	0.97 ± 0.05

endocardial level of activity in the area supplied by the circumflex branch. In all four dogs subjected only to "optimum" hemodilution the activity concentration was significantly higher in the epi- than endocardial region but only in areas supplied by the circumflex branch. Table II shows the mean ratios of epi- to endocardial activity concentrations of Xe-133 in these four dogs. The ratio is significantly higher in the region of circumflex supply than in the region supplied by the left anterior descending branch ($p < 0.005$ by method of

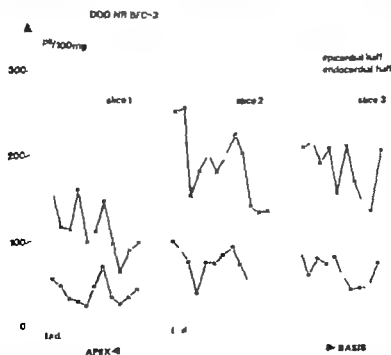


Fig. 4. The distribution of Xe-133 in the left ventricular wall of one dog plotted as in Fig. 2. Xenon was injected at a degree of hemodilution beyond optimum causing left ventricular insufficiency

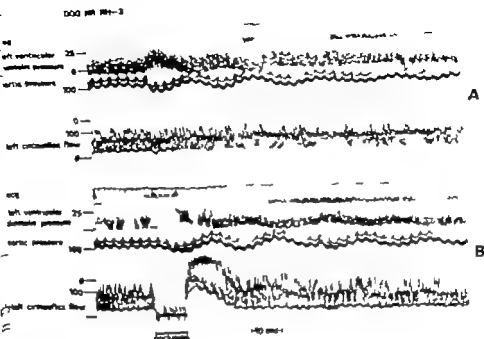


Fig. 5 Trache-Haring waves generated spontaneously (A) and during 10 second occlusion of the left coronary artery (B).

pared comparison). Compared to the ratio obtained in hemodiluted dogs with occluded left anterior descending branch (1.81 ± 0.11) the corresponding ratio is significantly lower ($p < 0.05$).

The last dog of group D was hemodiluted beyond the disappearance of the diastolic RH-response and until signs of left ventricular insufficiency appeared. Left ventricular diastolic pressure increased from 3 (before the onset of hemodilution) to 47 mmHg, aortic systolic and diastolic pressures decreased from 161 to 106 mmHg and from 136 to 86 mmHg respectively. "Diastolic" coronary flow was reduced to control level, while "systolic" flow was twice as high as the initial value. Fig. 4 shows the distribution of activity in the left ventricular free wall of this dog plotted as in Fig. 2. The ratio of epi- to endocardial activities as 3.11 ± 0.15 (SE), which is significantly higher than in the circumflex supplied area of optimum hemodiluted dogs with occlusion of the left anterior descending branch ($p < 0.01$).

In group E "systolic" and "diastolic" flows were increased by factors 4.6 (range 3.2-7.0) and 1.6 (range 1.3-4.0) respectively at peak reactive hyperemia. These factors are significantly different ($p < 0.005$ by method of paired comparison). Table II shows the mean ratios of epi- to endocardial activity concentrations in this group. The ratio for Xe 133 is significantly higher in the circumflex than in the left anterior descending area ($p < 0.05$ by method of paired comparison). Compared to the ratio in the circumflex area of hemodiluted dogs with unimpeded flow in the left anterior descending branch (1.38 ± 0.06) the corresponding ratio in group E is significantly lower ($p < 0.02$). The ratio of microsphere ac-

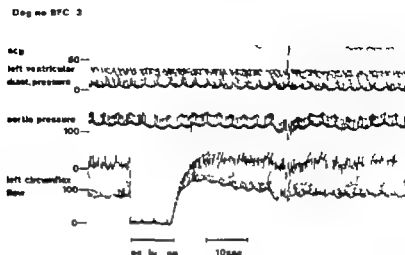


Fig. 6. Reactive hyperemic response in a hemodiluted dog with submaximum coronary vasodilatation. Note that the Traube-Hering waves only influence "diastolic" coronary blood flow

tivity concentrations in areas supplied by the left anterior descending and circumflex branches are not significantly different. Furthermore, they are not significantly different from the corresponding ratios for Xe 133

In some of the dogs of both groups (D and R) periods of fluctuations in arterial blood pressure appeared spontaneously and during the 10 s occlusions. These fluctuations, which had a frequency lower than that of the respiratory changes, are generally called vasomotor or Traube-Hering waves (TH waves) (e.g. Guyton and Harris 1951). The present expts.

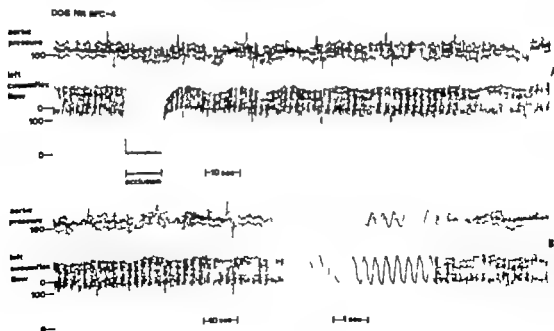


Fig. 7 Traube-Hering waves generated during 10 s occlusion of the left circumflex branch (A) and spontaneously (B) in a dog with "optimum" hemodilution. The pressure waves are reflected in both "systolic" and "diastolic" circumflex flow

seem to indicate a relation between the influence of these fluctuations on coronary blood flow and the degree of coronary vasodilatation. Therefore, the expts. in which they appeared will be examined more closely in this report. Fig. 5 shows TH-waves generated spontaneously (A) and during 10 s occlusion of the left coronary artery (B) in a normal, anesthetized dog. Although changes in arterial blood pressure produced by respiration were accompanied by oscillations in "systolic" coronary flow the TH-waves in A, apart from the initial, steep pressure decrease, did not influence "systolic" or "diastolic" coronary flow. When TH waves were present during reactive hyperemia (B), they were accompanied by oscillations in "diastolic" coronary flow while influence on "systolic" flow was not observed. In Fig. 6 coronary blood flow was recorded in a dog hemodiluted to a very small diastolic RH response, indicating almost maximum dilated coronary vessels. Also in this dog the TH-waves only produced fluctuations in "diastolic" coronary flow. Finally Fig. 7 shows that TH-waves at optimum hemodilution were reflected in both "diastolic" and "systolic" coronary blood flows.

Discussion

The activity concentrations of microspheres injected into the aortic root before hemodilution showed a uniform distribution in the left ventricular wall. This is in accordance with other reports about radioactive microspheres with a mean diameter of less than 15 μ m, injected into the left atrium (e.g. Buckberg *et al.* 1975) and with the distribution of Xe-133 after bolus injection into the aortic root of normal, anesthetized dogs (Bagger 1977 a). At "optimum" hemodilution and occlusion of the left anterior descending branch the activity concentrations of both Xe-133 and microspheres were significantly higher in the sub- than endocardial region. Furthermore, there was no difference between the ratios of sub- to endocardial activity concentrations of the two tracers. This seems to indicate that wash out of Xe-133 in the short period between injection of the isotope and cessation of coronary flow was negligible. Occlusion of the left anterior descending branch without hemodilution does not influence the distribution of Xe-133 in the circumflex supplied area (Bagger 1977 b). Therefore, the results indicate that the blood flow reserve is smaller in the sub- than epicardial region. This might explain why hemodilution was accompanied by a smaller increase in "diastolic" than "systolic" coronary blood flow. The present expts. were performed at maximum coronary vasodilatation accompanied by increased coronary blood flow and presumably modest decrease in perfusion pressure of the circumflex supplied region. This makes it unlikely that increased fractional loss of isotope from vessels supplying the endocardium during their passage through the epicardial area or a too slow stress relaxation of the subendocardial vasculature were the only reasons for the lower endo- than epicardial activity previously found during maximum vasodilatation with reduced coronary flow and perfusion pressure (Bagger 1977 b).

Because of the short or even absent main stem of the left coronary artery in dogs the flow transducer was placed on the left circumflex branch. Then hemodilution was continued until the vessels from this branch were dilated at a maximum defined by disappearance of the diastolic reactive hyperemic response. Spencer and Denison (1959) stated that the reliability of pulsatile flow measurements obtained with electromagnetic flowmeter depends on a good

contact between the electrodes and the vessel. This can only be achieved if the constricts the vessel, reducing its diameter about 15% (Meisner, Messmer and Hagl 1975). Such a stenosis will probably not cause a significant reduction in blood flow under normal conditions due to autoregulatory peripheral vasodilatation, but it will reduce the blood flow reserve of the supplied area. This will result in maximum vasodilatation of the area at a lower degree of hemodilution than otherwise necessary. In the present experiments, hemodilution was continued just until maximum vasodilatation was obtained in the circumflex supplied area. Assuming a small stenosis of the circumflex branch owing to the flow transducer, dilatation of the vessels from the left anterior descending branch was probably not a maximum, when the diastolic RH-response in the circumflex supplied area was abolished. This might explain the normal distribution of Xe-133 in the region supplied by the left anterior descending branch of hemodiluted dogs with unrestricted flow in this artery.

Progressive stenosis of the circumflex branch until maximum vasodilatation per se to the stenosis results in the dog in a collateral flow which is able to prevent endocardial hypoperfusion (Bagger 1977b). In the circumflex supplied region of "optimum" hemodilution dogs with unrestricted flow in the left anterior descending branch the ratio of epi- to endocardial activity concentrations was lower than that of the group with occlusion of the circumflex branch. This might indicate that collateral flow also participates in perfusion of the circumflex supplied area at "optimum" hemodilution. However, it was not able to prevent a relative endocardial hypoperfusion.

Provenza and Scherlis (1959) examined dog hearts and found that vessels supplying the endocardial region were of larger diameter than those supplying the epicardium. According to Downey *et al.* (1975) found a 40% higher endo- than epicardial activity of radioactive microspheres in perfused fibrillating dog hearts with maximum dilated coronary vessels while Cutarelli and Levy (1963) found a uniform distribution of Rb-86 in fibrillating hearts as long as the intraventricular pressure remained at a normal diastolic level. The present results indicate that even if there is a smaller endo- than epicardial diastolic vascular resistance at maximum vasodilatation, it can not fully compensate for the lower endo- than epicardial perfusion during systole.

Downey *et al.* (1975) found that coronary vasodilatation generated by infusion of papaverine into the left circumflex branch resulted in identical epi- and endocardial activity concentrations of radioactive microspheres. This contrast to the present experiments might be due to a difference between the degree of vasodilatation produced by papaverine and hemodilution hypoxia.

Salisbury, Cross and Rieben (1963) and Hirschorn and Kaiser (1970) found that increased left ventricular end diastolic pressure resulted in endocardial hypoperfusion presumably because of a decreased diastolic driving pressure. In the present experiments, left ventricular diastolic pressure was not influenced by "optimum" hemodilution (Table I). However, in one experiment, in which hemodilution was continued beyond "optimum" until left ventricular diastolic pressure increased, the relative endocardial hypoperfusion was accentuated and now present in both areas supplied by the two main branches of the left coronary artery.

Cyclic changes in arterial blood pressure at a frequency of 2-4 per min, so-called Traub-Hering waves, did not influence the coronary blood flow when they appeared spontaneous

in normal dogs (Fig. 5 A). As in the same recordings respiratory changes in arterial blood pressure were reflected in "systolic" coronary blood flow the lacking influence of the TH-waves seems to indicate synchronous fluctuations in coronary vascular resistance. These fluctuations would correspond to changes in peripheral vascular resistance caused by a varying sympathetic tone (Aalkjær 1935 Green, Lewis and Nickerson 1943). The fluctuating coronary vascular resistance is probably not due to autoregulation as this does not occur without any change in flow (Driscoll, Molr and Eckstein 1964). More likely it is a result of varying sympathetic influence directly on the coronary vessels. At maximum coronary vasodilatation elicited by blood dilution the TH-waves were reflected in both "systolic" and "diastolic" coronary blood flows (Fig. 7) indicating a disappearance of fluctuations in coronary vascular tone. In Fig. 6 TH-waves in a dog with submaximum coronary vasodilatation were accompanied by a fluctuating "diastolic" coronary flow while "systolic" flow was unaltered. This finding may be due to maximum vascular relaxation in the region mainly perfused during diastole (the endocardial part of the wall) and incomplete relaxation (and maintained responsiveness to sympathetic stimuli) in the region also perfused during systole (the epicardial part). This is in accordance with the conception of a smaller blood flow reserve in the endo- than epicardial region.

The results obtained after injection of Xe 133 and microspheres at peak diastolic reactive hyperemia showed a uniform distribution of both tracers in the epi- and endocardial regions of the left ventricular wall. This seems to indicate a uniform flow to these regions. Coronary occlusions of 10 s duration reduce myocardial contractility and systolic intramyocardial pressure (Van Der Meer 1972). Thus the uniform flow might be due to an improved endocardial perfusion during systole. This might also explain the larger increase in "systolic" than "diastolic" flow at peak reactive hyperemia. However this larger increase might also be explained by the larger epi- than endocardial blood flow reserve. Reneman and Spencer (1972) found that after 10 s occlusion of the left anterior descending coronary artery "systolic" reactive hyperemic flow reached its peak value sooner and declined faster than "diastolic" flow. The present expts. confirm these observations (e.g. Fig. 1 A). This might indicate a larger peak hyperemic flow and a shorter lasting hyperemic period in the epi- than endocardial region. It is therefore possible that at the time of the bolus injection, i.e. at peak diastolic reactive hyperemia, the reactive hyperemia, while still maximum in the endocardial region, might have declined in the epicardial. This might explain the uniform flow at peak diastolic reactive hyperemia and also why TH-waves present during reactive hyperemia only influence diastolic coronary flow detectably.

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The influence of scrotal warming on testicular blood flow and endocrine function in the rat

By

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Abstract

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The effects of local heating on testicular and epididymal vascular resistance in sodium pentobarbitone anesthetized rats was measured with microsphere techniques. When exposing the left scrotum to 33 and 41°C for 30 min, no significant effects on blood flows were observed in comparison to those of the right side. Exposure to 41°C caused significant ($p < 0.05$) decrease in vascular resistance of both testes and epididymides. The response was more pronounced at 43°C. The Leydig cell function, as judged from the testosterone concentrations in plasma and testicular tissue after LH stimulation, was significantly ($p < 0.01$) depressed at 41 and 43°C. It was concluded that the impaired Leydig cell function was correlated to testicular blood flow

In most mammals the scrotal temperature is lower than that of the peritoneal cavity and this difference in temperature is a prerequisite for a normal spermatogenesis. Impaired testicular function in cryptorchidism (for a review see, VanDenmark & Fries 1970) and varicocele (Greenberg 1977) is often attributed to an increased temperature. Although it has been shown that the secretion of testosterone is impaired in the congenital (Elk-Nes 1966) and experimentally (Damber *et al.* 1978) cryptorchid testis it is not clear to what extent the Leydig cells are dependent on low temperature for a normal sensitivity to gonadotrophic stimulation.

A possible explanation to the heat induced impairment of testicular function is disturbance of nutrition and oxygenation (Ewing & VanDenmark 1965, Walters & Setchell 1964) and thus, variations in testicular blood flow may be of importance. Several reports in various species indicate a moderate increase in testicular blood flow at temperatures above 40°C but hardly any response at temperature below this level (Glover 1966, Walters *et al.* 1968, Walters *et al.* 1973, Setchell *et al.* 1966). It has been shown that the experimentally cryptorchid testis in the rat has a relative increase in blood flow as compared to the scrotal one (Glover 1965, Damber *et al.* 1978). On the other hand, Ewing *et al.* (1963) reported a decreased flow of the perfused rabbit testis with increased temperature. A possible explanation to such discrepancies in results may be that testicular blood flow measurements are affected by the ex-

adrenals and some other organs as measured. Immediately after the injections of microspheres, the body temperature was measured through an abdominal incision. The vascular resistance of the various organs was calculated as mean arterial blood pressure/organ blood flow per unit of weight.

Testing of the Leydig cell sensitivity to LH. Forty rats were anesthetized and the tail artery was cannulated with polyethylene tubes (PE 30, Intramedic Clay Adams, USA. I.d. 0.6 mm, o.d. 1.0 mm) for sampling of arterial blood. Fatsen LU of heparin was given i.a. An arterial blood sample (0.6 ml) was then taken for determination of the basal testosterone level, immediately followed by an i.a. injection of 30 µg LH (NIH-B10). In each rat the scrotum was immersed in thermostat regulated water bath. Immersion of tail and hind legs was avoided. The rats were divided into 4 groups with 10 rats in each group, and the rats were exposed to 33, 37, 41 and 43°C, respectively for 30 min. Immediately after the scrotal warming the plasma testosterone determination, and the testes were removed, decapsulated and weighed. For testosterone analysis each testis was homogenized in 0.15 M NaCl.

Testosterone determination. All blood samples were centrifuged and plasma and tissue homogenates were stored at -20°C until analysis. The plasma samples were analyzed using radioimmunoassay previously verified (Damber & Jansson 1978b). From each testicular homogenate sample corresponding to 0.1 g of tissue was extracted 3 times with 5 ml chloroform. The combined chloroform extracts were washed with 1 ml 0.1 M NaOH, then twice with 2 ml distilled water and evaporated to dryness under stream of nitrogen in a water bath (40°C). Testosterone determination was performed using radioimmunoassay described by Damber & Jansson (1978).

Results

The body temperature in anesthetized rats was 36.9 ± 0.3 °C (mean \pm S.E.). The difference between scrotal and abdominal temperature was 3.7 ± 0.2 °C which was in agreement with the findings of Kormano (1967) in the conscious rat.

Effects on unilateral scrotal warming on testicular and epididymal vascular resistance

Warming of the left scrotum did not affect the temperature of the right testis. Table I shows that local scrotal warming elicited no significant effects on body temperature, blood pressure and on the distribution of blood flow to ventral prostate, kidneys and brain.

Table II shows a significant decrease ($p < 0.05$) in vascular resistance of testes and epididymis at temperatures of 41 and 43°C. The effect on the epididymis was more pronounced than that on the testis.

TABLE I The effect of unilateral scrotal warming on mean arterial blood pressure and vascular resistance in various organs in anesthetized rats

Left scrotal temperature (°C)	No.	Body temperature (°C)	Mean arterial blood pressure (mmHg)	Vascular resistance (mmHg/ml/100 g min)		
				Ventral prostate	Kidneys	Brain
33	6	37.1 ± 0.3	111.7 ± 4.4	5.6 ± 0.4	0.20 ± 0.02	3.0 ± 0.5
37	5	37.0 ± 0.4	110.8 ± 4.4	5.6 ± 0.7	0.27 ± 0.03	3.3 ± 0.6
41	6	37.2 ± 0.2	112.5 ± 4.4	5.6 ± 1.2	0.24 ± 0.03	2.8 ± 0.5
43	6	36.6 ± 0.3	102.5 ± 6.2	5.2 ± 0.6	0.18 ± 0.03	2.4 ± 0.7

Values are expressed as mean \pm S.E.

The significant differences between the groups for any parameter were achieved by analysis of variance.

perimental design, especially since scrotal heating above 40°C in a small animal like the rat has been shown to elicit profound effects on body temperature and several cardiovascular parameters (Waites *et al.* 1973) possibly concealing local effects on the testicular vasculature (Damber & Janson 1977).

The present study was undertaken to re-evaluate the effect of local heating on testicular blood flow in the rat using the microsphere technique. In addition, the gonadotropin sensitivity of the Leydig cells was tested *in vivo* at normal and supernormal temperatures.

Materials and methods

Animals. Male rats of the Sprague-Dawley strain (Anticimex Ltd, Stockholm, Sweden) weighing 360–430 g were kept in a controlled environment (temperature 25°C, light 05.00–19.00 h). Food and water were available *ad libitum*. The anaesthetic used was sodium pentobarbitone (Nembutal, Abbott, U.K.) given as a single dose of 40 mg/kg.

Measurements of scrotal and abdominal temperature. In 5 anesthetized rats iron-constantan thermocouples were placed in the right scrotum and in the middle part of the abdominal cavity through small skin incisions. The thermocouple was connected to a PM 421 II galvanometer (Philips).

Measurements of blood flow. In 4 anesthetized rats a midscrotal skin incision was performed and the left scrotal sac was introduced into a closely fitting copper basket which was surrounded by a heating jacket connected to a circulation thermostat. The heating device was kept in place by a thin rubber dome, holding the upper part of the device just above the cranial pole of the epididymis. The scrotal temperature was recorded by a thermistor connected to a Grass Model 7 Polygraph. The blood pressure and the distribution of blood flow was measured with the microsphere technique as described previously (Damber & Janson 1977). The spheres used were labelled with ^{51}Cr (mutual specific activity 13.54 mCi/g) and had a diameter of $14.5 \pm 1.1 \mu\text{m}$ (mean size \pm range). The experimental design is schematically illustrated in Fig. 1. The rats were divided into 4 groups (6 rats in each group) in which the left testis was exposed to 33, 37, 41 and 43°C, respectively. The right testis served as a control. After a period of 30 min the distribution of blood flow to the testis

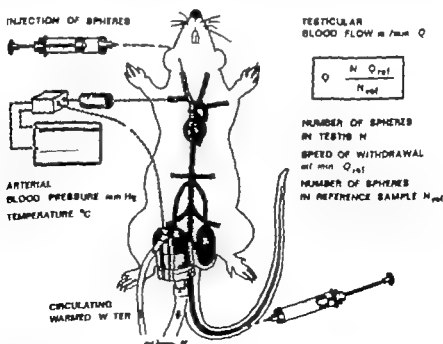


Fig. 1 Schematic illustration of the animal preparation. For details of the microsphere technique, see Damber & Janson 1977.

lymphatics and some other organs was measured. Immediately after the injections of microspheres, the body temperature was measured through an abdominal incision. The vascular resistance of the aorta was calculated as mean arterial blood pressure/organ blood flow per unit of weight.

Measuring of the Leydig cell activity in LH. Forty rats were anaesthetized and the tail artery was cannulated with polyethylene tube (PE 30, Intramedic Clay Adams, USA., I.d. 0.36 mm, o.d. 1.0 mm) for sampling of blood and i.a. injections. Fifteen I.U. of heparin was given i.a. An arterial blood sample (0.6 ml) as there was for determination of the basal testosterone level, immediately followed by an i.a. injection of 30 µg (NIH-B10). In each rat the scrotum was immersed in thermostat regulated water bath. Immersion of tail and hind legs was avoided. The rats were divided into 4 groups (10 rats in each group) and the rats were exposed to 33, 37, 41 and 43°C, respectively for 30 min. Immediately after the scrotal warming body temperature was measured through an abdominal incision, and another blood sample was collected for plasma testosterone determination, and the testes were removed, decapsulated and weighed. For testicular testosterone analysis each testis was homogenized in 0.15 M NaCl.

Testosterone determinations. All blood samples were centrifuged and plasma and deoxy homogenates were stored at -20°C until analysis. The plasma samples are analyzed using radio-immunoassay previously coded (Damber & Jansson 1978b). From each testicular homogenate sample corresponding to 0.1 g of testis was extracted 3 times with 5 ml chloroform. The combined chloroform extracts were washed with 1 ml 1 M NaOH, then twice with 2 ml distilled water and evaporated to dryness under stream of nitrogen in a water bath (40°C). Testosterone determination was performed using radio-immunoassay described by Damber & Jansson (1978).

Results

The body temperature in anesthetized rats was $36.9 \pm 0.3^\circ\text{C}$ (mean \pm S.E.). The difference between scrotal and abdominal temperature was $3.7 \pm 0.2^\circ\text{C}$ which was in agreement with the findings of Korman (1967) in the conscious rat.

Effects on unilateral scrotal warming on testicular and epididymal vascular resistance

The warming of the left scrotum did not affect the temperature of the right testis. Table I shows that local scrotal warming elicited no significant effects on body temperature, blood pressure and on the distribution of blood flow to ventral prostate, kidneys and brain.

Table II shows a significant decrease ($p < 0.05$) in vascular resistance of testes and epididymides at temperatures of 41 and 43°C. The effect on the epididymis was more pronounced than that on the testis.

TABLE I. The effect of unilateral scrotal warming on mean arterial blood pressure and vascular resistance in various organs in anesthetized rats.

Left scrotal temperature (°C)	No.	Body temperature (°C)	Mean arterial blood pressure (mmHg)	Vascular resistance (mmHg/ml/100 g min)		
				Ventral prostate	Kidneys	Brain
	6	37.1 ± 0.3	111.7 ± 4.4	5.6 ± 0.4	0.20 ± 0.02	3.0 ± 0.3
1	5	37.0 ± 0.4	110.8 ± 4.4	5.6 ± 0.7	0.27 ± 0.03	3.3 ± 0.6
1	6	37.2 ± 0.2	112.5 ± 8.4	5.6 ± 1.2	0.24 ± 0.03	2.8 ± 0.3
3	6	36.6 ± 0.3	102.5 ± 6.2	5.2 ± 0.6	0.18 ± 0.03	2.4 ± 0.7

Values are expressed as mean \pm S.E.

* Significant differences between the groups for any parameter were achieved by analysis of variance.

TABLE II The effects of a left-sided scrotal warming on testicular and epididymal vascular resistance (mmHg/ml/100 g · min) in anesthetized rats.

Left scrotal temperature (°C)	No	Testes			Epididymis		
		Right	Left	% Difference	Right	Left	% Difference
33	6	6.9 ± 1.4	7.6 ± 1.9	+ 5.6 ± 7.8	14.3 ± 2.3	13.7 ± 2.0	- 3.2 ± 4
37	5	7.3 ± 1.2	6.8 ± 1.0	- 8.2 ± 4.3	13.8 ± 1.9	12.5 ± 1.2	- 8.8 ± 1
41	6	8.3 ± 1.3	6.3 ± 1.3	- 28.6 ± 3.1	17.5 ± 3.4	10.0 ± 1.9	- 41.9 ± 3
43	6	7.3 ± 1.3	3.1 ± 0.8	- 36.2 ± 6.7	15.3 ± 3.4	3.1 ± 0.7	- 74.8 ± 7

Values are expressed as mean ± S.E.

Significant decrease ($p < 0.05$) of vascular resistance according to Wilcoxon's paired *t* test.

Effects of bilateral scrotal warming on the Leydig cell sensitivity to LH

The body temperature after 30 min of scrotal warming to different temperatures was 36.4 ± 0.1 °C and was not significantly different between the groups or from the basal levels (above). The effects of scrotal warming on plasma testosterone concentration before and after LH stimulation and the testicular testosterone concentration after LH treatment is shown in Table III. Fig. 2 illustrates the acute responsiveness of the testes to LH stimulation at increasing temperatures, expressed as the difference in plasma testosterone concentration before and 30 min after LH injection. The results clearly demonstrate that the Leydig cell function, expressed as the acute response to LH stimulation, is impaired at 41 and 43 °C.

Discussion

The moderate response in vascular resistance to increased scrotal temperatures found in the present study confirms the findings in the rat by Walters *et al.* (1973) and those in the ram Setchell *et al.* (1966). The experimental design to induce an increased testicular temperature did not evoke any alterations in body temperature and in any other cardiovascular parameter measured, indicating that the effect on testicular and epididymal vasculature is a local

TABLE III Effects of scrotal warming on plasma and testicular testosterone concentration 30 min after i.m. injection of 30 µg LH.

Scrotal temperature (°C)	No.	Plasma testosterone concentration (ng/ml)		Testis testosterone concentration (ng/g)
		Before LH treatment	After LH treatment	After LH treatment
33	8	5.3 ± 0.9	46.1 ± 2.9	2340 ± 85
37	8	6.2 ± 0.6	51.0 ± 3.4	2790 ± 118
41	10	3.0 ± 0.4	21.4 ± 2.1 ^a	830 ± 119 ^a
43	9	3.6 ± 0.3	9.8 ± 0.4 ^{a,b}	214 ± 21 ^{a,b}

Values are expressed as mean ± S.E.

^a Significant different from the groups with a scrotal temperature of 33 °C and 37 °C, $p < 0.01$ (Wilcoxon two-sample *t*-test).

^b Significantly different from the group with a scrotal temperature of 41 °C, $P < 0.01$.

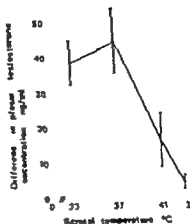


Fig. 2 Difference in plasma testosterone concentrations before and 30 min after 30 µg LH-1 at different scrotal temperatures. Bars indicate Mean \pm Standard Deviation. * significantly different from slices at 33°C. (p < 0.01) Wilcoxon two-sample t-test). † significantly different from slices at 41°C (p < 0.01).

one. The finding of no demonstrable change in testicular blood flow at 37°C supports the view that the increase in relative blood flow to the cryptorchid testis is due to morphological changes rather than to vasodilatation (Damber *et al.* 1978 c). The report by Ewing & VanDemark (1964) of decreased blood flow with increased temperature in the perfused rabbit testis was not supported by the results of the present measurements *in vivo*.

It has been shown in experiments with two different species (dog: Elk-Nes 1964, rat: Free & Talbot 1973, Damber & Janson 1978 a) that testosterone secretion is positively related to testicular blood flow at normal temperatures. In the present study a decrease in testicular sensitivity to LH and concomitant increase in testicular blood flow at supernormal temperatures indicates that the effect of heat on the Leydig cell function is not mediated by a primary effect on testicular perfusion as suggested by Ewing & VanDemark (1964). Collins & Lacy (1967) reported no change in interstitial steroid content after exposing rat testes to 43°C for 30 min. However it may well be that basal testosterone production is unaffected at short term exposures to heat while the capacity of the stimulated Leydig cell to produce/secrete androgens is affected as shown in the present study. It is interesting to note that the same temperatures (41°C) which impairs the Leydig cell function at short term exposures is known to reduce sperm output from the rat testis (Setchell & Walters 1972) and testicular weight (Mann & Walters 1977). It is tempting to speculate that the reduction in sperm output and testicular weight is due to an impaired Leydig cell function. Since it has been shown that the Leydig cell function is impaired in the experimentally cryptorchid testis of the rat (Damber *et al.* 1978), it is possible that long term exposure to an abdominal temperature elicits the same type of endocrine changes as short term exposures to supernormal temperatures. The effect on testicular function following experimentally induced cryptorchidism or heat treatment appears to be dependent on the duration of exposure as well as on the temperature of exposure (VanDemark & Free 1970).

The mechanism responsible for the decreased sensitivity of the Leydig cells to LH stimulation may be heat induced changes in their metabolism. Hall (1965) found a maximal effect of LH stimulation on rabbit testicular protein and testosterone synthesis *in vitro* at 38°C, which

TABLE II The effects of a left-sided scrotal warming on testicular and epididymal vascular resistances (mmHg/ml/100 g · min) in anesthetized rats.

Left scrotal temperature (°C)	No.	Testes			Epididymis		
		Right	Left	% Difference	Right	Left	% Difference
33	6	6.9 ± 1.4	7.6 ± 1.9	+ 5.6 ± 7.8	14.3 ± 2.3	13.7 ± 2.0	- 3.2 ± 4.4
37	5	7.5 ± 1.4	6.8 ± 1.0	- 8.2 ± 4.3	13.8 ± 1.9	12.5 ± 1.4	- 8.8 ± 8.5
41	6	8.5 ± 1.5	6.3 ± 1.3	- 28.6 ± 3.1	17.5 ± 3.4	10.0 ± 1.9	- 41.9 ± 5.3
43	6	7.3 ± 1.5	3.1 ± 0.8	- 56.2 ± 6.7	15.3 ± 3.4	3.1 ± 0.7	- 74.8 ± 7.9

Values are expressed as mean ± S.E.

Significant decrease ($p < 0.05$) of vascular resistance according to Wilcoxon's paired *t*-test.

Effects of bilateral scrotal warming on the Leydig cell sensitivity to LH

The body temperature after 30 min of scrotal warming to different temperatures was $36.4 \pm 0.1^\circ\text{C}$ and was not significantly different between the groups or from the basal levels (see above). The effects of scrotal warming on plasma testosterone concentration before and after LH stimulation and the testicular testosterone concentration after LH-treatment are shown in Table III. Fig. 2 illustrates the acute responsiveness of the testes to LH stimulation at increasing temperatures, expressed as the difference in plasma testosterone concentration before and 30 min after LH injection. The results clearly demonstrate that the Leydig cell function, expressed as the acute response to LH stimulation, is impaired at 41 and 43°C .

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The moderate response in vascular resistance to increased scrotal temperatures found in the present study confirms the findings in the rat by Waltes *et al.* (1973) and those in the ram by Setchell *et al.* (1966). The experimental design to induce an increased testicular temperature did not evoke any alterations in body temperature and in any other cardiovascular parameter measured, indicating that the effect on testicular and epididymal vasculature is a local

TABLE III Effects of scrotal warming on plasma and testicular testosterone concentration 30 min after i.v. injection of 30 µg LH

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43	9	5.6 ± 0.5	9.8 ± 0.4 ^{a,b}	214 ± 21 ^{a,b}

Values are expressed as mean ± S.E.

Significant different from the groups with a scrotal temperature of 33°C and 37°C , $p < 0.01$ (Wilcoxon two-sample *t*-test).

^a Significantly different from the group with a scrotal temperature of 41°C , $P < 0.01$.

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is the normal scrotal temperature in this species. In addition, LeVier & Spaziani (1968) found in experiments with unstimulated rat testicular tissue *in vitro* an inhibition of protein synthesis concomitant with a reduction in steroid conversion, at temperatures above and below normal scrotal levels. Heat induced changes in testicular glucose metabolism may also be of importance (Setchell & Hinks 1967, Davis & Langford 1970, Deb *et al.* 1977).

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Studies on calcium uptake by myometrial microsomes with particular reference to the dependence on inorganic phosphate and oxalate

By

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Abstract

BATRA S. *Studies on calcium uptake by myometrial microsomes with particular reference to the dependence on inorganic phosphate and oxalate*. Acta physiol. scand 1978, 104: 68-73.

Ca uptake by microsomes isolated from non-pregnant rabbit myometrium was potentiated by both inorganic phosphate (P_i) and oxalate anions. Both P_i and oxalate had little effect on the initial rate of uptake but a pronounced effect on the capacity of Ca uptake measured after 20 min which was greater in the presence of oxalate than that of P_i (5 mM each). The presence or absence of sucrose in the uptake medium had a significant effect on oxalate-induced potentiation of Ca uptake but not on that potentiated by P_i that measured in the absence of either potentiating anion. A part of Ca accumulated additionally and the influence of sucrose could be removed by washing microsomes with KCl.

Another significant difference between the potentiating effect of oxalate and P_i was observed when the pH of the incubation medium was varied. In the presence of oxalate the pH optimum was between 6.4-6.8 whereas that in the absence or in the presence of P_i the optimal pH was around 7.2. Reduction in pH from 7.2 to 6.8 along with the substitution of KCl by sucrose resulted in 3-fold increase in Ca uptake when oxalate was used as the potentiating anion. The results suggest that Ca is taken up by different mechanism in the presence of oxalate than that in its absence or when oxalate anion is substituted with inorganic phosphate.

Since the demonstration of an ATP-dependent Ca uptake in isolated microsomal and mitochondrial fractions from the myometrium (Batra and Daniel 1969; Batra and Daniel 1971 a, Carsten 1969; Batra and Daniel 1971 b) Ca uptake by subcellular fractions isolated from several types of smooth muscles has been reported (see Janis and Daniel 1977 for references). Ca uptake by the microsomal fraction of smooth muscle differed not only quantitatively from that of skeletal muscle (Batra 1975) but certain qualitative differences in particular the lack of stimulation by oxalate of Ca uptake by smooth muscle microsomes were also noted (Batra and Daniel 1971 a, Baudouin-Legros and Meyer 1973, Zelick *et al.* 1974).

975 Worcel *et al.* 1976). In some other studies, however, ovalate stimulation in microsomes isolated from certain smooth muscles (Fitzpatrick *et al.* 1977, Hurwitz *et al.* 1973, Ford and Hess 1975, Godfraind *et al.* 1976, Ruysschaert *et al.* 1977) could be demonstrated. Although Ca uptake in some of these recent investigations (Ford and Hess 1975, Godfraind *et al.* 1976, Ruysschaert *et al.* 1977) was measured by Millipore-filtration technique (Martoson and Feretos 1964), the retentate (material on the filter) rather than the filtrate was counted for Ca^{45} -radioactivity determinations. This modification, which has also been recently made in the measurements of mitochondrial Ca uptake (Jacobus *et al.* 1975) makes an important difference in that Ca uptake without unspecific (or loose) binding is measured. This is achieved by washing the material on the filters before the determination of the retained isotopic Ca. Furthermore, when low amounts of Ca are taken up, the reliability of the method measuring uptake directly in the retentate, rather than by the difference in the filtered and unfiltered samples, is better.

Since inorganic phosphate (P_i) is a physiological anion, occurring in relatively high concentrations in the myoplasm, we have, in the present study, examined its effects on Ca uptake kinetics in rabbit myometrial microsomes and compared them with those of ovalate. Not only are we able to clearly demonstrate the potentiation by P_i as well as by ovalate but provide some evidence showing that the two anions increase Ca accumulation by different mechanisms.

Methods

Preparation of microsomes

Non-pregnant New Zealand White rabbits weighing between 2.7 to 3.2 kg. are killed by cervical dislocation. The uterine horns are removed and placed in Krebs-Ringer bicarbonate-solution bubbled continuously with 95% O_2 and 5% CO_2 . The composition of the Krebs-Ringer medium was (mM): 115 NaCl, 4.0 KCl, 0.1 CaCl_2 , 1.16 MgSO_4 , 1.16 NaH_2PO_4 , 21.9 NaHCO_3 and 49 mM glucose.

Each horn was trimmed of excess fat and connective tissue, cut open longitudinally and endometrium removed by scraping. The uterine horns are kept in bubbling Krebs-Ringer solution throughout this period. The scraped myometrium is blotted dry and placed in ice-cold sucrose (0.25 M) and Hepes (10 mM) solution, pH 7.2. The tissue, after weighing, is homogenized in about 10 volumes of the above sucrose-Hepes solution with Polytron homogenizers for 10 \times 3 periods (per g tissue) with intermittent pauses of 20 s. The homogenate was centrifuged at 1000 \times g for 10 min and the pellet discarded. The supernatant was filtered through 3 layers of gauze and centrifuged at 15 000 \times g for 15 min. The resulting pellet, which was enriched in mitochondria, is occasionally used for experiments dealing with Ca uptake measured in mitochondria. The above supernatant was centrifuged at 40 000 \times g for 1 h to obtain the microsomal pellet. The pellet was suspended in sucrose-Hepes solution to give a protein concentration of 1-2 mg/ml and used for Ca uptake experiments immediately. Protein concentration in the microsomal fractions was determined by the method of Lowry *et al.* (1951).

Measurement of Ca uptake

Microsomes were incubated at 37°C in 1 ml of solution containing (mM): 20 Hepes buffer (pH 7.25), 4 ATP, 5 MgCl_2 , 5 $\text{Na}_2\text{S}_2\text{O}_8$ and 0.025 CaCl_2 including Ca^{45} . The medium was supplemented with sucrose 0.2 M or KCl 0.1 M or NaCl 0.1 M. Microsomal protein concentration was 80-120 μg per ml. After incubation, 0.2 ml of the reaction mixture was filtered at various times under suction through Millipore filters (HAWP 04500). The filters were subsequently washed with 2 ml of Hepes buffer containing sucrose or KCl or NaCl depending on the composition of the reaction mixture. The radioactivity retained by the filters was counted by liquid scintillation spectrometry using Aquasol (New England Nuclear Corp.) as the scintillation fluid. Control tubes with the complete incubation mixture but without the microsomal proteins were incubated in each assay. Control filters showed less than 0.05% of the Ca^{45} present in the reaction mixture irrespective of its composition.

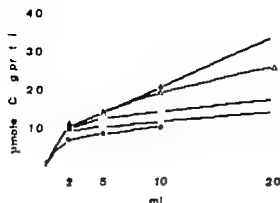


Fig. 1

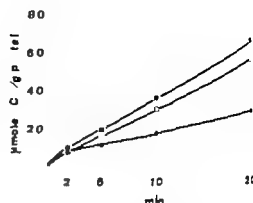


Fig. 2

Fig. 1. Ca uptake by rabbit myometrial microsomes in the presence of 2 mM P_i (■), 5 mM P_i (Δ), 2 mM oxalate (□), 5 mM oxalate (▲), and in the absence of potentiating anions (●). Uptake medium was supplemented with KCl.

Fig. 2. Ca uptake by rabbit myometrial microsomes from a medium supplemented with KCl (●) or sucrose (■). The middle curve represents data (□) of Ca uptake from a medium supplemented with sucrose. Microsomes after Millipore filtration were washed with KCl (see Methods).

Results

The effect of P_i on the time course of Ca uptake by rabbit myometrial microsomes is shown in Fig. 1. Whereas 2 mM oxalate had no significant effect on Ca uptake, the same concentration of P_i increased Ca uptake slightly but significantly. Both oxalate and P_i had little effect on the initial rate of uptake but a pronounced effect on the capacity of Ca uptake, measured after 20 min, and Ca uptake after this period was greater in the presence of 5 mM oxalate than that of 5 mM P_i . When NaN was omitted from the medium, Ca uptake increased by only about 20% (not shown).

The presence of sucrose in the uptake medium had a significant effect on oxalate-induced potentiation of Ca uptake but not on that potentiated by P_i or on that measured in the absence of either potentiating anion (Table I). There was no significant difference in Ca uptake when the medium was not supplemented or was supplemented instead of sucrose with KCl or NaCl or choline chloride. This was true in both the absence and presence of potentiating anions indicating that sucrose increased potentiation by oxalate rather than the cations depressed it.

TABLE I. The effect of sucrose or electrolytes added as supplements to the reaction mixture on Ca uptake by rabbit myometrial microsomes. Ca uptake was determined after 20 min of incubation. Values are means \pm S.E. of those obtained in 4 or 5 separate experiments.

Supplement	Ca uptake (μ mol/g prot l)		
	without oxalate	with oxalate	with P_i
None	7.88 ± 1.0	23.15 ± 2.7	21.50 ± 2.4
Sucrose	10.66 ± 1.3	58.46 ± 4.8	26.76 ± 2.3
KCl	8.36 ± 1.2	20.39 ± 2.4	21.42 ± 2.0
NaCl	9.57 ± 0.9	19.96 ± 2.1	22.38 ± 2.3
Choline chloride	12.88 ± 1.2	19.51 ± 1.7	26.54 ± 2.4

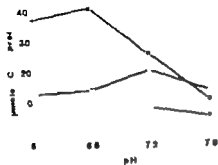


Fig. 3

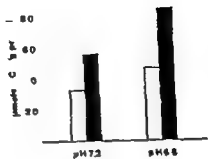


Fig. 4

Fig. 3. Dependence on pH of Ca uptake in the absence of potentiating anions (O), or in the presence of P_i (●) or oxalate (●).

Fig. 4. Effect of pH on oxalate-potentialized Ca uptake from medium supplemented with KCl (open bars) or sucrose (closed bars).

Microsomes that had accumulated Ca from a sucrose containing medium in the presence of oxalate lost some of the bound Ca when washed with KCl instead of sucrose solution (Fig. 2), but the amount of Ca remaining in the microsomes was still considerably higher than that found after the accumulation from a KCl medium.

Another significant difference between the potentiating effect of oxalate and P_i was observed when the pH of the incubation mixture was varied (Fig. 3). In the absence of potentiating anions a pH change between 6.4 to 7.6 had little effect on Ca uptake. In the presence of P_i the maximum uptake was observed at pH 7.2 although the pH-optimum had a relatively broad peak. When oxalate was used as a potentiator of Ca uptake, the pH-optimum was 6.8 but even at 6.4 the uptake was near maximum. However at pH 7.2 Ca uptake decreased by about 50% and was not significantly different than that observed with P_i at this pH. Increasing pH to 7.6 further reduced oxalate-supported Ca uptake.

Having observed that a reduction in pH or the inclusion of sucrose in the medium increased oxalate-supported, but not P_i -supported, Ca uptake considerably the effect of sucrose at lower pH (6.8) was examined. As seen in Fig. 4 lowering the pH had an additive effect to that observed by inclusion of sucrose in the medium. Thus, a reduction in pH from 7.2 to 6.8 along with the substitution of KCl by sucrose almost tripled the amount of Ca taken up.

Discussion

The present results clearly demonstrate a potentiation by both oxalate and P_i of Ca uptake by myometrial microsomes. Rabbit myometrial microsomes prepared and assayed in the way as described here were also found to have much higher affinity for Ca uptake (Batra 1977) than that reported previously by us (Batra 1973, Batra 1975). Furthermore, a Ca-stimulated ATPase activity related stoichiometrically to Ca uptake, could also be demonstrated (to be published).

The potentiating effect of P_i on Ca uptake by the microsomal fraction of smooth muscle was not unexpected but has not been demonstrated previously. The present data clearly show that potentiation by P_i of Ca uptake differs not only quantitatively but also qualitatively from that observed with oxalate. Oxalate potentiation of Ca uptake in contrast to that by P_i was sensitive to the composition of the medium as it was highly stimulated by the presence of sucrose in the medium as well as by lowering of pH below neutrality. The effects of increasing H^+ ion concentration and the presence of sucrose were additive. Whether this stimulation is simply due to a greater permeability of oxalate to microsomal membranes under these conditions or whether it involves a more complicated mechanism cannot be known. In any event the present results showing a pronounced stimulation of Ca uptake by sucrose or by a reduction in pH specifically in the presence of oxalate might provide an explanation for some of the controversy on the oxalate potentiation of Ca uptake in smooth muscle microsomes.

Our results on the pH influence on oxalate stimulated uptake are in agreement with the recently published by Godfraind *et al.* (1976) on intestinal smooth muscle microsomes. Krall *et al.* (1976) using a relatively alkaline (pH 8) reaction mixture recently reported oxalate potentiation of Ca uptake by rat myometrial microsomes. However the rates of Ca uptake in their study were extremely low and uptake did not reach a plateau even after 30 min of reaction which is in contradiction with our previous data (Batra and Daniel 1971) as well as with those of Nishikori *et al.* (1977) reported recently. In fact, the data of Krall *et al.* showed practically no ATP-dependent uptake in the first 5 min whereas the data of the other two studies mentioned above (Batra and Daniel 1971 a, Nishikori *et al.* 1977) showed that Ca uptake reached a plateau after 5 min. In view of this it seems difficult to correlate the kinetics of Ca uptake presented by Krall *et al.* with the physiological function. No explanation was given by the authors for either these extremely slow rates of Ca uptake or the use of a reaction medium at pH 8.

The present results also showed that a part of the Ca taken up under the influence of sucrose in the presence of oxalate could be released by KCl, but not by sucrose, washing. This would indicate some Ca binding at the surface of the vesicles for which K^+ can compete with Ca^{++} . However there is a fraction of Ca which is probably transported inside the vesicle under the influence of sucrose as KCl washing removed only 39% of additionally accumulated Ca. The study of the mechanism of this action of sucrose deserves further attention. Interestingly sucrose has been shown to antagonize the inhibition by certain drugs and elevated temperature of Ca uptake by cardiac sarcoplasmic reticulum (Solomon *et al.* 1972). Since not only the effect of pH but also of sucrose was specific for oxalate, it is suggested that Ca is taken up by a different mechanism in the presence of oxalate than in its absence or when oxalate anion is substituted with inorganic phosphate.

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Oxidative and lysosomal capacity in skeletal muscle of mice after endurance training of different intensities

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Abstract

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The activity of certain enzymes of energy metabolism (cytochrome c oxidase, citrate synthase, malate dehydrogenase and lactate dehydrogenase) and of lysosomes (β -glucuronidase, β -N-acetylglucosaminidase, arylsulphatase, ribonuclease, deoxyribonuclease, acid phosphatase and cathepsin D) was assayed in *m. rectus femoris* of mice trained 5 days per week, 1 h per day for 4 weeks according to 4 different programmes. I. running speed 20 m/min, horizontal track. II. 25 m/min, horizontal track. III. 20 m/min 8° up hill inclination, and IV. 25 m/min 8° uphill inclination. Oxidative capacity increased and anaerobic capacity decreased without distinction between the different training programmes. Of acid hydrolases shown the activities of β -glucuronidase and cathepsin D were increased independently of training intensity. Simultaneous histochemical observations on β -glucuronidase and arylsulphatase activities in β contralateral *m. rectus femoris* showed more intense staining in red as compared to white muscle fibres. It is suggested that training affected the red fibres and that the applied level of loading was probably too low to cause major involvement of white fibres.

Key words. Acid hydrolases, skeletal muscle endurance training, training intensity, oxidative capacity, mouse.

Adaptations in the functional capacities of skeletal muscles occur according to the type of physical activity exercised. Thus prolonged exercises, like running or swimming, increase the mitochondrial or oxidative capacity (e.g. Holloszy and Booth 1976) whereas high intensity strengthening exercises, e.g. weight-lifting, increase the capacity of the contractile apparatus (e.g. Jaweed *et al.* 1974).

The type, duration and intensity of exercise as well as the fibre type profile of muscle involved in performance are generally accepted as factors of major importance influencing the magnitude and nature of responses occurring in the exercised muscles. Recent attention has been paid to the quantitative aspects of these factors in endurance exercise (Fitts *et al.* 1975, Terjung 1976, Dohm *et al.* 1977). In these investigations the activities of energy metabolism enzymes have been under the study.

The activities of some lysosomal hydrolases are also affected by physical exercise. The activity of β -glucuronidase is increased in crude skeletal muscle homogenates of endurance-trained mice (Viikio *et al.* 1974, Pihstrom *et al.* 1978). The following investigation was aimed to study the effects of different training intensities on the activity and localization of certain acid hydrolases and on activity of certain enzymes of energy metabolism.

Material and methods

Animals and training

all 83 male NMRI mice, aged 70–80 days at the beginning of training, were used in the experiments. Mice training the mice were randomly divided into 5 groups: I. Untrained controls ($n = 22$) living under normal cage conditions, II. A group (TR 20/H) ($n = 15$) running on horizontal track of motor driven treadmill at speed of 20 m/min for 1 h per day, III. A group (TR 25/H) ($n = 16$) running on horizontal track at speed of 25 m/min for 1 h per day, IV. A group (TR 30/U) ($n = 16$) running uphill (inclination 8°) speed of 20 m/min for 1 h per day, and V. A group (TR 25/U) ($n = 16$) running uphill (inclination 8°) speed of 25 m/min for 1 h per day. The animals were exercised 5 days/week. During the first 8 days training the running time was increased from 30 to 60 min and speed from 12 to 20 or 25 m/min simultaneously and equally for each group until the desired speed was obtained. The exercise remained at this level 16–18 days and was terminated the day before the mice were killed. Two motor driven treadmills, one for horizontal and the other with an uphill track, were used in the training. The animals were exercised at 10–11 h in groups TR 20/H and TR 20/U, 11–12 h in groups TR 25/H and TR 25/U, and 12–13 h in group TR 30/U each day. Day in the cages was artificially divided into 12 h of light and 12 h of darkness changing at 08.00 h. The mice were housed 7 or 8 mice to a cage (Type IV Scanbur Denmark), and they had free access to solid food pellets (Astra-Ewos R 3, Sæboen) and tap water. The normally growing control mice, feeding ad lib, gained more weight during the study (Table I) as compared to all the trained groups. The continuous earlier observations made with rats (Teyssie 1976). The temperature (21–22°C) and humidity (50–60%) were automatically kept constant in the animal house.

Mice preparation and assay methods

Animals were killed by dislocation of the neck. Skin was removed and *an. quadriceps femoris* dissected and further divided into separate muscles of which *an. rectus femoris* was studied. The muscle was immediately weighed. No differences in the weights between trained and control groups were observed (Table I). The sample was homogenized in ice-cold homogenization medium (150 mM KCl, 50 mM KHCO₃, and 6 mM EDTA, pH 7.4) in Potter Elvehjem homogenizer with glass pestle. The homogenates (3 w/v) were kept at 18°C until analyzed within two weeks. Before assaying the activity of lysosomal enzymes an aliquot of this preparation was made 0.1 in respect to Triton X 100 concentration in order to determine total activity of lysosomal hydrolases.

Activities of the following acid hydrolases were assayed: β -glucuronidase (β -GLuc) (EC 3.2.1.31), L-N-acetylglucosaminidase (β -GlcNAc) (EC 3.2.1.30), p-nitrophenylphosphate ("acid phosphatase") (p-NPPase) (EC 3.2.3.2), cathepsin D (Cat. D) (EC 3.4.4.23), arylsulphatase (ASul) (EC 3.1.4.1), ribonuclease (RNase) (EC 2.7.7.16), and deoxyribonuclease (DNase) (EC 3.1.4.6). Acid hydrolase assays were performed essentially as described by Barrett (1972).

The activities of cytochrome oxidase (CytOx) (EC 1.9.3.1), malate dehydrogenase (MDH) (EC 1.1.1.37), and citrate synthase (CitSy) (EC 4.1.2.7) were used as measures of the oxidative capacity of the muscle and lactate dehydrogenase activity (LDH) (EC 1.1.1.27) as a measure of anaerobic capacity of muscle. CytOx was estimated polarographically with Clark electrode (Wharcat *et al.* 1969), MDH and LDH according to Ochoa (1953) and Kornberg (1953), respectively after appropriate dilution of the homogenate and CitSy according to Stern (1969). Protein content (Table I) was determined after hydrolyzing 0.25 ml homogenate with 10 ml 1 N NaOH at 40°C for 60 min according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard. The enzymatic activities at 37°C (acid hydrolases), 25°C (MDH, CitSy and LDH) or at 28°C (CytOx) were referred per muscle wet weight.

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TABLE II. The activities of cytochrome *c* oxidase (CytOx), citrate synthase (CytS), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) in the thigh muscle (*m. rectus femoris*) of four groups of mice trained according to different training programmes. Activities are expressed as nmol substrate consumed/min/g muscle wet weight at 25 °C, except for CytOx at 28 °C. Statistical significance as in Table I.

group		CytOx	CytS	MDH	LDH
Control	22	14.09 ± 2.47	31.77 ± 3.21	235.3 ± 34.4	366.7 ± 61.2
Horizontal, 20 m/min	13	18.68 ± 2.43	38.92 ± 5.81	314.6 ± 40.7	340.0 ± 67.7
Horizontal, 25 m/min	14	18.20 ± 3.16	39.01 ± 6.74	329.7 ± 50.4	324.0 ± 63.4
Uphill, 20 m/min	16	19.35 ± 3.25	40.55 ± 4.31	331.9 ± 43.0*	337.1 ± 53.4
Uphill, 25 m/min	16	19.34 ± 2.52	41.20 ± 5.73	343.6 ± 47.5	333.4 ± 60.6
✓ All trained	61	18.84 ± 2.81	39.97 ± 5.60	331.6 ± 43.4	333.8 ± 61.2
□ All horizontal	29	18.45 ± 2.74	38.96 ± 6.05	332.0 ± 44.7	332.3 ± 66.1
✓ All uphill	32	19.20 ± 2.82	40.88 ± 4.83	340.4 ± 44.2	335.3 ± 56.2

histological and histochemical observations

Glucuronidase activity was more prominent in red muscle fibres, especially in their myofibrillar area, as compared to white fibres in which only minor activity was sometimes seen in inter-fibrillar space (Fig. 1 B). Activity granules appeared longitudinally arranged between myofibrils as also in the case of arylsulphatase activity (Fig. 1 C). β -Glucuronidase activity was only occasionally seen outside muscle cells. Arylsulphatase staining appeared only in red fibres and some cells (probably fibroblasts) seen between fibres (Fig. 1 C D). In histological samples some degenerative and regenerative fibres were seen both in control and in trained animals.

Histochemical and histological examination did not reveal any major quantitative or qualitative differences between the trained (TR 25/U) and control groups.

Discussion

In the present study the duration of daily exercise was set to 1 h due to previous experience with mice, which showed that moderate 1 or 2 h daily running exercise caused a similar

TABLE III. The activities of β -glucuronidase (β -GUase), β -acetylglucosaminidase (β -GAase), arylsulphatase (ASase), p-nitrophenylphosphatase (p-NPPase), ribonuclease (RNase), deoxyribonuclease (DNase) and cathepsin D (CatD), in the thigh muscle (*m. rectus femoris*) of four groups of mice trained according to different training programmes. Activities are expressed as pmol/min/g muscle wet weight at 37 °C. Statistical significance as in Table I.

group		β -GUase	β -GAase/ASase	RNase	DNase	Cat.D	p-NPPase	
Control	22	6.74 \pm 0.77	235 \pm 53	29.3 \pm 5.2	301 \pm 30	190 \pm 20	83.4 \pm 24.9	1.242 \pm 78
□ Horizontal, 20 m/min	13	7.96 \pm 1.11	** 239 \pm 43	23.0 \pm 2.9	286 \pm 53	193 \pm 18	99.3 \pm 26.4	1.241 \pm 91
□ Horizontal, 25 m/min	14	8.98 \pm 0.77*	246 \pm 51	22.1 \pm 2.3	320 \pm 48	199 \pm 16	95.4 \pm 20.4	1.291 \pm 95
□ Uphill, 20 m/min	16	7.86 \pm 0.93	240 \pm 52	29.6 \pm 4.7	319 \pm 43	200 \pm 17	97.9 \pm 30.2	1.266 \pm 100
□ Uphill, 25 m/min	16	9.11 \pm 4.53	238 \pm 65	27.5 \pm 5.8	304 \pm 53	191 \pm 28	94.1 \pm 25.9	1.238 \pm 92
✓ All trained	61	8.47 \pm 2.43	243 \pm 52	27.6 \pm 4.4	307 \pm 49	196 \pm 20	96.7 \pm 25.3	1.258 \pm 94

TABLE I Weights of experimental animals and their *rectus femoris*-muscle together with protein or of the *rectus femoris*-muscle in control group and four different trained groups. Mean \pm standard deviation. The significance of differences. $p < 0.05$, $p < 0.01$ and $p < 0.001$

Group	n	Weight of animals (g)	Weight of the <i>rectus</i> -muscle (mg)	Protein content of the <i>rectus</i> -muscle (mg, mg)
I Controls	22	39.5 \pm 3.6	107.3 \pm 14.3	179.3 \pm 16.0
II Horizontal, 20 m/min	15	35.6 \pm 2.5	103.1 \pm 14.6	182.7 \pm 12.0
III Horizontal, 15 m/min	14	37.0 \pm 3.0	110.3 \pm 10.3	180.7 \pm 9.3
IV Uphill, 20 m/min	16	37.4 \pm 2.4	110.0 \pm 8.5	180.7 \pm 13.0
V Uphill, 25 m/min	16	36.5 \pm 2.7*	105.0 \pm 10.5	180.0 \pm 11.3

Bird 1970), 2 representative acid hydrolases (β -GUase and ASase) were stained histochemically in cryostat sections. A *rectus femoris* from the contralateral leg to the part used for biochemical assays removed, cut into two parts which were embedded on specimen block with OCT-compound (Fisher Tech, Ames) for longitudinal and cross sectioning. Samples were frozen in isopentane prechilled by liquid nitrogen. Serial cryostat sections for the staining of NADH-dehydrogenase (Novikoff *et al.* 1961), β -oxidase (Chayen *et al.* 1973), and cytochrome (Goldfisher 1972) activities and for hematoxylin-staining were cut with Ames Cryostat II and mounted on cover glasses. On the basis of NADH-dehydrogenase activity individual muscle cells were classified either as red or white fibres, the latter having low oxidative capacity respectively. Histochemical analysis was performed only on control and TR 25/U groups.

Statistical methods

Standard procedures were used to calculate means and standard deviations (SD). The statistical significance of differences between the means of control and the trained groups were tested by Student's *t*-test for non-correlating means.

Results

Enzymes of energy metabolism

The activities of all estimates of aerobic metabolism (CytOx, CitSy and MDH) were statistically significantly increased due to the different training programmes (Table II). This increase in oxidative capacity was in the mean of 30%. On the contrary LDH activity slightly decreased, the decrease being significant only when all values of trained animals were pooled. Depending on work intensity slightly higher increases in oxidative capacity were observed after uphill training than after horizontal training. This effect, like the effect of running speed, was not statistically significant.

Acid hydrolases

Training increased β -GUase activity in all exercised groups regardless of intensity (Table III). Systematically higher values in trained groups were found in Cat D activity. This increase was significant ($p < 0.05$) only for the pooled values of trained animals. In other activities (β -GAase, RNase, DNase, and p-NPPase) no significant differences were observed between control and trained animals. ASase activity decreased in group II (TR 20/H).

The protein concentrations of *m. rectus femoris* were similar in all animal groups (Table I).

Available data on anaerobic or lysosomal capacity do not permit similar conclusions as to influence of daily exercise time.

Benzi *et al.* (1975) suggest that the magnitude of the adaptation in oxidative capacity (succinate dehydrogenase, cytochrome oxidase and NADH cytochrome c reductase activities) in rat gastrocnemius and soleus muscles is dependent both on daily work load and on total running time. Daily work load was varied both in respect to the duration and speed of running. The design of their study does not permit evaluation of the effects of speed or running time separately. In a study by Dohm *et al.* (1977) with rat gastrocnemius the changes in daily running speed during a steady-speed endurance training programme of 6 weeks did not significantly affect the activity of succinate dehydrogenase, and the data of Terjung (1976) show slight and insignificant increases in cytochrome c concentration and citrate synthase activity in rat gastrocnemius as a result of raising the elevation of the treadmill.

In agreement with Dohm *et al.* (1977) and Terjung (1976) the present results (CytOx, LDH, and CitSy) show slight but insignificant effects of speed or higher intensity caused by treadmill elevation on the oxidative capacity of the rectus femoris muscle of mice. Neither the training changes in anaerobic (LDH activity) or lysosomal capacity (Table III) significantly affected by the intensity of training.

Adaptation of the oxidative capacity of mixed skeletal muscle to moderate endurance training seems thus to be more affected by the length of the daily exercise period (Fitts *et al.* 1975, Dohm *et al.* 1977), if the duration is below a hypothetical threshold value (approximately 2 h for the rat, 1 h or less for the mouse), than by relatively slight changes in intensity (speed or extra load caused by treadmill elevation). If, however, both duration and speed are increased simultaneously the response in oxidative capacity is stronger (Benzi *et al.* 1975).

The muscle under study (*ss. rectus femoris*) is a mixed skeletal muscle, therefore our results represent the average of all heterogeneous adaptive responses, which have occurred in the three, metabolically different, fibre types. Moderate endurance training programmes result in an increase in the oxidative or respiratory capacity of all three (slow-twitch red, slow-twitch red, and fast-twitch white) skeletal muscle fibre types in rat (Baldwin *et al.* 1972) and similarly Sahmen *et al.* (1977) showed an increase both in predominantly red and predominantly white parts of the quadriceps femoris muscle of mice. Increases in oxidative capacity after moderate endurance training have always been more pronounced, in chemical terms, in red as compared to white muscle tissue, although relatively they may be almost equal in the two tissue types both in the rat and the mouse (Baldwin *et al.* 1972, Sahmen *et al.* 1977). There seems also to be a tendency for a higher increase of β -nucleosidase activity in red muscle tissue compared to white in mice as a response to moderate endurance exercise programmes (Vihko *et al.* 1977 a).

Glycogen depletion studies by Armstrong *et al.* (1974) with rats showed that contractile activity at low work intensities is maintained by oxidative fibres (both slow and fast twitch), and that major use of anaerobic fibres (fast-twitch glycolytic type) occurs at high work intensity (increased running speed) or after depletion of glycogen stores in oxidative fibres. In contrast to gastrocnemius muscle (mixed) Terjung (1976) observed an effect of

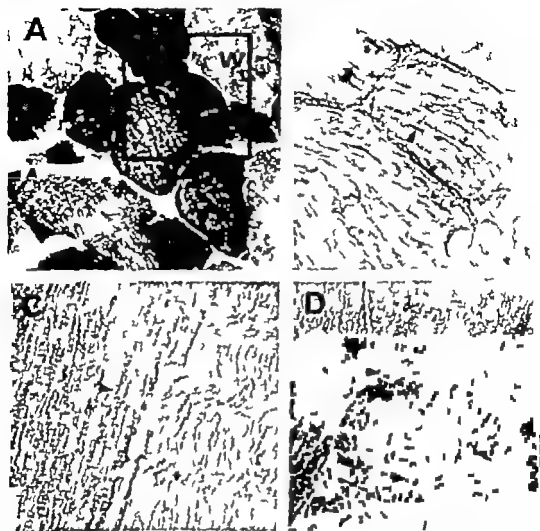


Fig. 1. A. NADH tetrazolium reductase ("diaphorase") staining showing the classification of fibres into highly oxidative red (darkly stained) and low oxidative white fibres (moderately or slightly stained). Sections from control animal. Magnification 290. B. Serial section for β -glucuronidase staining from control animal, outlined area from Fig. 1 A. Slight activity in control samples was usually seen in red fibres in this case (arrows) but usually not in white fibres (w). Occasional activity was now and then seen also in muscle fibres. Magnification 675. C. An example of arylsulphatase staining, trained animal. Activity is typically seen only in red, highly oxidative fibres (left). Magnification 875. D. Arylsulphatase staining from control animal. In this case some activity is also seen in structures outside muscle fibres in addition to typical activity in red, highly oxidative fibres. Magnification 625.

response in the activity of MDH or β -GUase (Vihko *et al.* 1974). With rats Fitts *et al.* (1975) reported an increase in oxidative capacity (cytochrome c concentration, citrate synthase activity and malate-pyruvate oxidation rate) up to 2 h daily running exercise. Terjung (1976) showed that running for longer periods than 2 h per day did not further increase the oxidative capacity (cytochrome c concentration and citrate synthase activity) of rat gastrocnemius. Thus, at least with rodents, the influence of the duration of daily exercise on the oxidative capacity of the working "mixed" muscles during moderate endurance training at a steady speed is such that maximal adaptive response is achieved after a certain threshold time and further exercise of equal intensity is not effective.

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higher treadmill incline on markers of oxidative capacity in fast twitch white (white) muscle fibres indicating an increased involvement of white muscle with increasing work load. The intensity factors (speed and higher running incline) thus especially affect white muscle.

The training effects of the present experiment on oxidative capacity in mouse muscle (femoris-muscle) were evidently directed to red fibres, and the upper range of intensity (fast running speed) was too low to cause continuous recruitment of white muscle fibres.

The physiological significance of increased β -glucuronidase or cathepsin D activity in moderate endurance training is not clearly understood. The rest level activity of these and some other acid hydrolases is higher in predominantly red as compared to white skeletal muscle tissue in mice (Vihko *et al.* 1978 a). Moderate endurance training or one exercise bout also affects β -glucuronidase activity more in red than in white muscle (Vihko *et al.* 1978 a, b). The general concept of the functions of lysosomal hydrolases (de Duve 1973) suggests that increased activities parallel increased catabolism.

Increased activity of β -glucuronidase might be a reflection of a changed catabolic level in the homeostasis of endurance trained muscle. Cathepsin D activity correlates with leucine- 14 C incorporation rate in human skeletal muscle (Lundholm and Schersten 1975). Enhanced anabolism, which in trained muscle is obvious on the basis of e.g. increased mitochondrial capacity, might be compensated by increased lysosomal activity. Histochemical observations reported here show that biochemically found increases in β -glucuronidase activity probably occur inside muscle fibres and not e.g. in connective tissue cells as we have previously suggested (Vihko *et al.* 1974, Piiistö *et al.* 1978). The training-induced increase in β -glucuronidase activity seems to be more pronounced in red, oxidative muscle fibres (Vihko *et al.* 1978 a). The involvement of red muscle cells during moderate, prolonged endurance training probably is thus also demonstrated by the activity of β -glucuronidase.

The hypothesis of increased lysosomal capacity in connection with increased contractile activity of skeletal muscle was supported by the activities of β -GUase and Cat.D whereas the activities of β -GAase, RNase, DNase and p-NPPase were unchanged and ASase activity even decreased after the lightest training programme. Any definite explanation for the differentiation of training-induced adaptations (Table III) in catabolic activities, which usually function as an entity (de Duve 1973) cannot be given, although differentiation may simply reflect the strictly directed effects of endurance training on the catabolic capacity of muscle fibres.

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The mechanisms underlying these specific effects on the action potential and the receptor potential have been discussed in terms of detergent-membrane interactions (Jørgensen 1977). Both detergent-lipid and detergent-protein interactions seem to be involved. Although experimental findings favour the idea of a detergent-protein effect, at least the decrease in the fast sodium current in nerve and muscle, it has not been possible to completely rule out either alternative.

The frog neuromuscular junction, where acetylcholine binds to the nicotinic acetylcholine receptor protein, thereby inducing a depolarization in the postsynaptic membrane, is well characterized both electrophysiologically (Katz 1966) and morphologically (Birks *et al.* 1960, Cottman *et al.* 1970, Dreyer *et al.* 1973, Heuser *et al.* 1974). Furthermore, receptor proteins from electric organs have been purified and characterized biochemically (see Bergman *et al.* 1975, Hellstrom 1976). This makes the end-plate a suitable preparation for pharmacological-toxicological studies and in particular for studies of effects on the postsynaptic cholinergic processes in isolation by iontophoretic application of acetylcholine. The present study was undertaken in order to further examine the action of Triton detergents. The action of Triton X-43 and Triton X 100 on different processes involved in neuromuscular transmission was investigated. In particular the effect on the postsynaptic membrane, i.e. the acetylcholine receptor was studied. Electrophysiological measurements were combined with ultrastructural observations of the muscle end-plate, in order to establish if morphological changes were produced by Triton detergents.

Methods

The experiments were performed on isolated cutaneous pectoris muscles of the frog (*Rana temporaria*). This muscle was chosen because it is very thin (only about three layers of muscle fibres) and consequently the effect of the detergent to the end-plate region should be optimal (cf. Dreyer *et al.* 1974). The muscles were dissected free and placed with its deep surface up-ward in a petri dish (volume 2 ml). The normal Ringer solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 , 2.15 mM Na_2HPO_4 and 0.65 mM NaH_2PO_4 , pH being 7.0-7.2 (Adrian 1936). All experiments including the fixation procedure were performed at room temperature (22° - 24°C).

Electrophysiological recordings. The experimental procedures were in part the same as described earlier (Jørgensen 1977). Intracellular membrane potential changes were recorded with glass capillary microelectrodes filled with 3 M KCl, resistance about 10 M Ω and tip potential less than 5 mV. Microelectrodes used to inject current were filled with 2 M K-citrate, resistance 15-20 M Ω . The microelectrodes used to apply acetylcholine iontophoretically were filled with 2 M acetylcholinechloride (Kjellm) the resistance being 30-40 M Ω .

The recording microelectrode was connected to a high input impedance amplifier ($>10^9$). The output from the amplifier was fed into an oscilloscope (Tektronix 5103 N), a digital voltmeter (to display resting membrane potential) and an amplifier (Tektronix AM 302) which could be either DC (0-3 000 Hz) or AC-coupled (0.1-3 000 Hz). The output from this amplifier was usually set so that total amplification of 200 times was achieved and was used to record spontaneous miniature end-plate potentials (m.e.p.p.'s) or iontophoretically induced end-plate potentials. The 10 and 300 outputs were in addition fed into two channels of a tape recorder (Hi-Fi and Packard 3949). The recording speed was 3 3/4 inch/s which is equivalent to a bandwidth of 0-1 250 Hz. The membrane potential changes thus stored on tape could later be displayed on the oscilloscope and recorded on photographable film. The current passed through the muscle fibre or the iontophoretic microelectrode (see below) could be monitored on the oscilloscope as the voltage drop across the feedback resistor in an operational amplifier circuit used to hold the bath at virtual ground potential. This signal was also fed into one channel of the tape recorder via a 250 DC amplifier.

Acetylcholine was ejected by applying positive voltage pulses to the pipette. In some experiments small

Triton detergents and the frog neuromuscular end-plate **An electrophysiological and ultrastructural study**

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Abstract

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The effects of the nonionic detergents Triton X-45 and Triton X 100 were studied in the frog muscle end-plate by intracellular recordings of spontaneous miniature end-plate potentials (m.e.p.p.s) and the potential changes produced by iontophoretic application of acetylcholine (ACh-potentials). In addition, the ultrastructural changes produced by Triton X 100 were studied by transmission electron microscopic and freeze-fracture techniques. It was found that Triton X-45 and Triton X 100 caused a rapidly developing reduction of the amplitude of the m.e.p.p.s. The response to iontophoretic application of acetylcholine was reduced by Triton X 100. Following return to normal Ringer solution the ACh-potentials recovered although not completely. The dissociation constant calculated from the rate constants for onset and offset of the reaction ($k_D = k_{off}/k_{on}$) was 5-50 μ M depending on the type of stoichiometric reaction presumed to occur between Triton X 100 and the cholinergic receptor. The ultrastructural changes observed indicate that the nerve terminal plasma membrane and mitochondria are affected by Triton X 100. Leakage of Ca^{++} from the latter may therefore be the cause of the increase in m.e.p.p. frequency. It is concluded that the influence on the amplitude of the m.e.p.p.s and the ACh-potentials can be attributed to a direct effect of the detergent upon the acetylcholine receptor protein.

Detergents of the nonionic Triton series, in particular Triton X-45 and Triton X 100 have been shown to have specific effects on the excitability of nerves and muscle. The action potential in frog skeletal muscle fibre is suppressed, the maximum rate of rise of the impulse being reduced to 50% by Triton X 100 ($40 \cdot 10^{-6}$ M) (Rydqvist 1977). These results and work on frog myelinated nerve fibres (Brisman and Rydqvist 1978) have provided evidence that the suppression of the action potential is most likely due to an effect on the fast initial increase in sodium permeability which underlies the action potential in nerve and muscle (Hodgkin *et al.* 1952, Adrian *et al.* 1970). It has also been demonstrated that Triton detergents block the action potential in crustacean stretch receptor neurons and that prolonged exposure reduces and finally abolishes the receptor current. This block of the transducer action of the neuron may be attributed to a specific action on the sodium permeability increase during stretch as revealed by voltage clamp analysis (Ottoson and Rydqvist 1978).

The mechanisms underlying these specific effects on the action potential and the receptor potential have been discussed in terms of detergent-membrane interactions (Rydqvist 1977). Both detergent-lipid and detergent-protein interactions seem to be involved. Although experimental findings favour the idea of a detergent-protein effect, at least or the decrease in the fast sodium current in nerve and muscle, it has not been possible to completely rule out either alternative.

The frog neuromuscular junction, where acetylcholine binds to the nicotinic acetylcholine receptor protein, thereby inducing a depolarization in the postsynaptic membrane, is well characterized both electrophysiologically (Katz 1966) and morphologically (Birks *et al.* 1960, Cotman *et al.* 1970, Dreyer *et al.* 1973, Heuser *et al.* 1974). Furthermore, receptor proteins from electric organs have been purified and characterized biochemically (see Chang *et al.* 1975, Heffron 1976). This makes the end-plate a suitable preparation for pharmacological-toxicological studies and in particular for studies of effects on the postsynaptic cholinergic processes in isolation by iontophoretic application of acetylcholine. The present study was undertaken in order to further examine the action of Triton detergents. The action of Triton X-45 and Triton X 100 on different processes involved in neuromuscular transmission was investigated. In particular the effect on the postsynaptic membrane, *i.e.* the acetylcholine receptor was studied. Electrophysiological measurements were combined with ultrastructural observations of the muscle end-plate, in order to establish if morphological changes were produced by Triton detergents.

Methods

*The experiments were performed on isolated caudate muscles of the frog (*Rana temporaria*).

*The muscle was chosen because it is very thin (only about three layers of muscle fibres) and consequently

*diffusion of the detergent to the end-plate region should be optimal (cf. Dreyer *et al.* 1974). The muscles

*were dissected free and placed with its deep surface up and in perspex chamber (volume 2 ml). The normal

*Ringer solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 2.13 mM Na₂HPO₄ and 0.85 mM

*NaH₂PO₄, pH being 7.0-7.2 (Adrian 1956). All experiments including the fixation procedure were performed at room temperature ($22^{\circ} \pm 2^{\circ}\text{C}$).

*Electrophysiological recordings. The experimental procedures are in part the same as described earlier

(Rydqvist 1977). Intracellular membrane potential changes were recorded with glass capillary micropipettes

*filled with 3 M KCl, resistance about 10 M Ω and tip potential less than 5 mV. Micropipettes used to inject

*current were filled with 2 M K-chrome, resistance 15-20 M Ω . The micropipettes used to apply acetyl-

*choline iontophoretically was filled with 2 M acetylcholinechloride (Aldrich) the resistance being 30-40 M Ω .

The recording micropipette was connected to high input impedance amplifier (10^9). The output from

the amplifier was fed into an oscilloscope (Tektronix 5M320), digital volt-meter (to display resting mem-

*brane potential) and an amplifier (Tektronix AM 502) which could be either DC (0-3 000 Hz) or AC

*coupled (0.1-3 000 Hz). The output from this amplifier was usually set so that total amplification 1 200

*times was achieved and was used to record spontaneous miniature end-plate potentials (m.e.p.p.) or ionto-

*phoretically induced end-plate potentials. The 10 and 200 outputs were in addition fed into two

*channels of tape recorder (Hewlett and Packard 3949). The recording speed was 3 3/4 inch/s which is

*equivalent to bandwidth of 0-1 250 Hz. The membrane potential changes thus stored on tape could later

*be displayed on the oscilloscope and recorded on photographic film. The current passed through the muscle

*fibre or the iontophoretic micropipette (see below) could be monitored on the oscilloscope as the voltage

*drop across the feedback resistor in an operational amplifier circuit used to hold the bath at virtual ground

*potential. This signal was also fed into one channel of the tape recorder via 250 DC amplifier.

*Acetylcholine was ejected by applying positive voltage pulses to the pipette. In some experiments a small

negative (usually less than 1 volt) DC potential was applied to the inside of the pipette in order to inhibit diffusion of acetylcholine out of the pipette. The positive pulses used to release ACh had an amplitude of 1 to 6 V with a duration of 4 ms. The current produced by the applied voltage ranged between 0.3–2 nA and could be monitored by the voltage current converter described above. The duration and the current used are equivalent to a charge of $0.1\text{--}1.0 \times 10^{-6}$ Coulomb. The current pulses were usually not displayed during the experiment. The depolarization response due to release of ACh from the micropipette is referred to as "ACh potentials" (del Castillo and Katz 1955).

A superficial end-plate was located with the aid of a 100 stereo microscope fitted with dark field illumination and the recording microelectrode inserted into the fibre. If the resting membrane potential was stable for about 5 min and the miniature e.p.p. of moderate amplitude the iontophoretic pipette was carefully approached to the same region and placed within 100 μm from the recording microelectrode. With small relocations it was usually possible to find a spot where ACh-potentials could be released by repetitive pulses delivered every 14 s. This interval between pulses was found not to desensitize the end-plate (Thesleff 1953, Katz *et al.* 1956). If the ACh-potentials and the recording conditions in general remained stable for about 30 min the actual experiment was started. In many fibres either the resting membrane potential deteriorated or the ACh potential was not stable. In these cases the fibres were rejected. The number of fibres which were used to follow effects of long-time exposure of Triton detergents were limited because small fibres which are known to have large m.e.p.p.'s sometimes depolarized after relatively short periods of time following insertion of the microelectrode. On the other hand, fibres which were large as judged from the small m.e.p.p.'s were more difficult to use for accurate measurements of both m.e.p.p. amplitude and frequency.

Application of substances was performed by adding about 1 ml of the test solution and a subsequent withdrawal of the same volume. This was made three times for each substance to be tested. In this way the solution in the bath could be changed to about 90% in about 30 s, without dislodgement of the electrodes. In particular the iontophoretic electrode was very sensitive to fluid movements in the bath and even with careful performed changes of solution this pipette was sometimes moved in relation to the muscle fibre as judged from the change in amplitude of the ACh-potentials. Such fibres were only used to follow the change in miniature end-plate potentials (m.e.p.p.'s) provided the recording electrode was still inside the cell.

Electron microscopy Cutaneous pectoris muscles were carefully dissected free and pinned down in a resting length in two identical chambers. One muscle was treated with the detergent solution (Triton X 100 160×10^{-6} M) the other kept as a control. Muscles with visibly damaged fibres were not used. All fixation procedures were performed at room temperature ($+22$ – $+24^\circ\text{C}$). After treatment with the detergent usually for 30 min, both muscles were fixed for 60–90 min. The fixative used was either 2.5% glutaraldehyde and 2% paraformaldehyde or 3% glutaraldehyde and 5% paraformaldehyde in Na-cacodylate buffer at pH 7.1–7.3 to which 2 mM CaCl_2 and 2 mM MgCl_2 was added. Buffer solutions of concentration of 0.05 to 0.10 M were used. After fixation small pieces (about 0.5 mm) rich in end-plates were cut out from the muscle and postfixated for 60 min in 2% OsO₄ in the corresponding buffer. After rinsing in buffer the specimens were mordanted with 1% tannic acid according to Samsonescu and Samsonescu (1976). After dehydration in alcohol and embedding in Epon, ultrathin sections were cut on an LKB-ultramicrotome stained with uranylacetate (Watson 1958) and lead citrate (Reynolds 1963) and examined in a Zeiss EM10 electron microscope. All solutions used were freshly prepared except for the OsO₄ solution which was used within one week after preparation. To obtain freeze-fractured replicas the same primary aldehyde fixation were used. End-plate rich regions (about 0.5 mm) were cut out and equilibrated in 30% glycerol for 30 min at room temperature. The tissue was frozen in Freon 22 (monochlorodifluoromethane) cooled to -144°C by liquid nitrogen, fractured at -100°C and replicated with a 60 etching in Balzers 360 M apparatus fitted with an electron beam gun for Pt/C shadowing and a quartz crystal monitor to provide standardized replica thickness of about 2.0 nm. Replicas were cleaned in Na-hypochlorite $\text{H}_2\text{O}-\text{H}_2\text{SO}_4-\text{H}_2\text{O}$ and mounted on formvar coated copper grids. Illustrations are positive images where regions of platinum deposits are dark while absence of platinum appears light.

Triton X-45 and Triton X 100 were gifts from Rohm & Haas Co (Sweden) and used without further purification. These detergents belong to a series of homologues with the general formula $\text{C}_{18}\text{H}_{33}\text{O}_2$, $\text{C}_8\text{H}_{17}\text{O}$ ($\text{CH}_2(\text{CH}_2\text{O})_n\text{H}$) (Triton surface active agents). The nonionic octylphenoxy-ethanol (OPE) series, C8-40 (Rohm & Haas Co, Philadelphia), n is 8 and Triton X-43 (M W 426) has a mean n -value of 4.5 and Triton X 100 (M W 626) has a mean n -value of 9.8 (Handbook of Physical Properties, C8-Ole Rohm & Haas Co, Philadelphia, Samson *et al.* 1973). The hydrophilic lipophilic balance number (HLB) is 10.4 for Triton X-45 and 13.5 for Triton X 100.

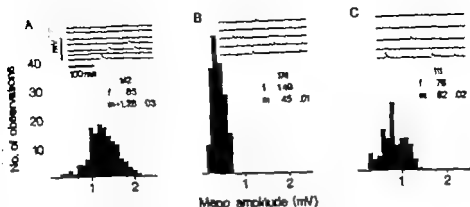


Fig. 1. Effect of Triton X 100 ($160 \cdot 10^{-6}$ M) on the amplitude and frequency of m.e.p.p.s. Histograms from a distribution of m.e.p.p. amplitudes before (A) and after 13-15 min in Triton X-100 (B). At about 15 min the fibres are returned to normal Ringer solution and after an additional 30 min m.e.p.p. amplitudes are recorded (C). Insets show representative traces of m.e.p.p.s. m means \pm S.E. of m.e.p.p. amplitude in mV, cs mean m.e.p.p. frequency in s^{-1} , number of m.e.p.p.s. Resting membrane potential: A - 95 mV, B - 13 mV, C - 87 mV.

Results

The effects of Triton detergents on the amplitude and frequency of the spontaneous m.e.p.p.s were first studied. Membrane potential and input resistance (R_i) were also followed. In a series of experiments iontophoretic application of acetylcholine was performed and change in response studied as a result of Triton X 100 treatment. The time course of onset and offset of the reaction between the detergent and the end-plate was used to calculate the dissociation constant (K_d). Prostigmin (10^{-6} g/ml, $3.3 \cdot 10^{-6}$ M) was used in some experiments but no effect on the action of the detergent was observed.

Effect on spontaneous miniature end-plate potentials (m.e.p.p.s). A series of experiments was carried out to study the effect of Triton X 100 and Triton X-45 at different concentrations on the miniature end-plate potentials (m.e.p.p.s). It was found that both Triton X-45 and Triton X-100 in concentrations of 80 - $160 \cdot 10^{-6}$ M (cf Rydqvist 1977) reduced the amplitude of the m.e.p.p.s within 5-30 min, the time course varying with concentration and also from preparation to preparation. In general $160 \cdot 10^{-6}$ M of both detergents invariably abolished the m.e.p.p.s after about 15 min. The difference in effect between source fibres is most likely due to variations in the diffusion barriers at the end-plate, as some of the end-plates studied might have been located on the inferior side of the fibre. At lower concentrations, e.g. $40 \cdot 10^{-6}$ M, the time required to obtain clear effects was considerable, and these concentrations were therefore not used in the present study. In Fig. 1 is shown the distribution of amplitude and the frequencies before, during and after application of Triton X-100 ($160 \cdot 10^{-6}$ M). This fibre was chosen to illustrate typical detergent effects because the m.e.p.p.s were not completely abolished and it was thus possible to construct amplitude histograms and to calculate mean m.e.p.p. frequencies. As seen the amplitude is reduced to about one third of the control amplitude after 13-15 min. After return to normal Ringer solution (at 15 min) the amplitude increased but did not

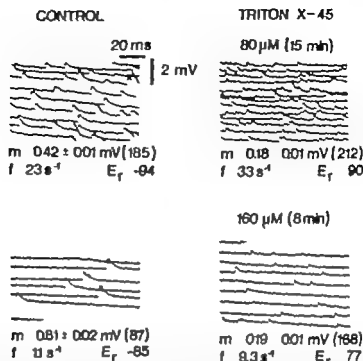


Fig. 2. Records of m.e.p.p.s from two fibres showing the effect of Triton X-45 at two concentrations (80 and 160×10^{-6} M). From the recordings in normal Ringer solution (control) and in detergent solutions the mean \pm S.E. (m) and the frequency (f) of the m.e.p.p. are calculated using additional traces not shown in the picture. Numbers in brackets indicate number of m.e.p.p.s used for these calculations. The resting membrane potential (E_r) in mV. Prostaglandin 10^{-6} g/ml.

return to control value. As shown in earlier work on frog muscle action potential (Rydqvist 1977) the time needed to complete recovery is considerable. It is probable that this may partly be ascribed to deterioration of the muscle fibre or to factors which are not dependent on the detergent treatment *per se*.

The frequency of the m.e.p.p.s was seen to increase from a control value of 0.8 s^{-1} to 1.5 s^{-1} after about 15 min in Triton X 100. After return to normal Ringer solution the frequency decreased and attained a value of 0.8 s^{-1} identical to that of the control. The increase in frequency was seen in most fibres treated with both Triton X-45 and Triton X 100 as is illustrated in Fig. 2 and Fig. 3. These figures show the effect of Triton X-45 and Triton X 100 respectively on the amplitude and frequency of the spontaneous activity of different end-plates. The increase in frequency due to the detergents is best illustrated at 160×10^{-6} M Triton X-45 (Fig. 2) while in Fig. 3 there is no increase at 80×10^{-6} M and at 160×10^{-6} M the m.e.p.p.s are almost completely abolished so that an estimate of the frequency is impossible. As is also evident in Fig. 2 and Fig. 3 there is no basic difference between the effect of Triton X-45 and Triton X 100 which is in accordance with earlier work on frog muscle and on the crustacean stretch receptor (Rydqvist 1977, Ottoson and Rydqvist 1978). As the variability of the frequency increase was considerable the effect of Triton on the frequency of the spontaneous activity was studied on five fibres treated with Triton X 100, 160×10^{-6} M. End-plates were chosen for which the spontaneous m.e.p.p.s were only partially suppressed. The results are outlined in Table I and it can be seen that there is a consistent

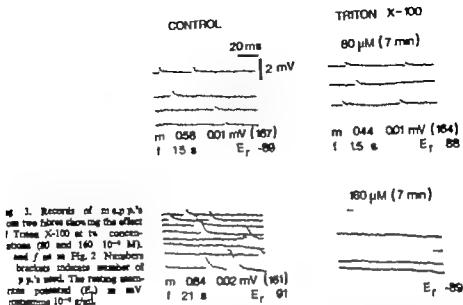


Fig. 1. Records of m.e.p.p.s on two fibres showing the effect of Triton X-100 at two concentrations (80 and 160 10^{-4} M), and f as in Fig. 2. Numbers in brackets indicate number of p.p.s. used. The resting membrane potential (E_r) in mV ranges 10^{-4} g/l.

of variable increase in frequency. This variability can in part be explained by the fact that when the m.e.p.p. amplitude is decreased some m.e.p.p.s will be hidden in the noise and thus the value of the frequency in Triton solution is most likely underestimated.

The time course of individual m.e.p.p.s is seen to be little affected by Triton detergents. As judged from Fig. 2 and Fig. 3, a small increase in the time constant for the decay might be present. This difference was not further examined in the present study.

In addition to direct effects of the Triton detergents on the neuromuscular transmission, the resting membrane potential and the input resistance is known to affect the amplitude of the m.e.p.p.s (Katz *et al.* 1957a). The equilibrium potential for the active end-plate is about 15 mV and the m.e.p.p. is then proportional to E (5, *i.e.* the driving force of the p.p., where E is the absolute value of the actual membrane potential. The amplitude of the m.e.p.p.s is also proportional to the input resistance (R_i) of the muscle fibre. It has earlier been shown that the membrane resistance is little affected by Triton X 100

TABLE 1. Increase in m.e.p.p. frequency after exposure to Triton X 100 (160 μ M). Numbers in brackets indicate resting membrane potential.

Fibre	Time of exposure (min)	Frequency	
		Control ($n=9$)	Triton X-100 160 μ M ($n=9$)
I	12	6.6 (-65)	31.6 (-63)
II	6	8.6 (-92)	10.8 (-92)
III	0.55 (-89)	0.55 (-89)	1.49 (-81)
IV	5	3.63 (-80)	6.8 (-77)

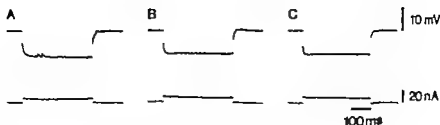


Fig. 4 Effect of Triton X 100 on electrotonic potential changes (upper trace) in response to rectangular current pulses (lower trace) recorded from a muscle end-plate. The current electrode was inserted less than 100 μ m from the recording electrode.

A: in normal Ringer solution I put resistance (R_i) = 1.37 M Ω .

B: after 15 min in Triton X 100 (160 μ M), R_i = 1.20 M Ω .

C: after 30 min in Triton X 100 (160 μ M), R_i = 1.1 M Ω .

Resting membrane potential in A = -70 mV, B = -59 mV and in C = -53 mV.

(Rydqvist 1977) but in order to exclude any gross changes in R_i during exposure at high concentrations of detergents R_i was followed in 5 fibres after they had been exposed to 160×10^{-6} M Triton X 100, *i.e.*, the highest concentration used in the present experiment. In Fig. 4 is shown the membrane potential changes due to a 10 nA current pulse in normal Ringer solution (A) and after 15 min (B) and 30 min (C) in 160×10^{-6} M Triton X 100. The electrodes were positioned about 100 μ m from each other and the value of R_i is thus only an approximation of the true value, which is calculated by rectangular pulse analysis (*cf.* Boethius and Rydqvist 1977). The method is, however, useful when comparing effects over a limited time period. It should be noted, however, that R_i cannot be used to estimate changes in the specific membrane resistance (R_m) because of the unknown length constant (λ).

As can be calculated from Fig. 4 $R_i = (V/I)$ in this fibre decreased from a control value of 1.37 M Ω to 1.12 M Ω after 30 min. In 5 cells R_i was decreased by a mean of 14% (range 0–18%) which should imply a decrease in m.e.p.p. amplitude of the same amount. In a few cells the R_i was followed after the cell had been returned to normal Ringer solution but only small changes in R_i were seen even when the cell was soaked for up to 60 min. The changes in the resting membrane potential and the R_i are insufficient to explain the decrease in the amplitude of the m.e.p.p.s which must consequently be due to a specific action on the neuromuscular transmission.

Effect on ACh-potentials

The results presented so far suggest that Triton detergents cause a block of the transmission at the end plate. This raises the question whether this is a presynaptic or a postsynaptic effect. It is evident that presynaptic effects must exist from the fact that the frequency of the spontaneous m.e.p.p.s is increased. The decrease in m.e.p.p. amplitude on the other hand can be explained as both pre- and postsynaptic effects. To study the influence of detergent (Triton X 100) on the postsynaptic membrane, iontophoretic application of acetylcholine to the end plate was performed. To ensure that the electrodes were reasonably well in place, several precautions were taken, some of which are described

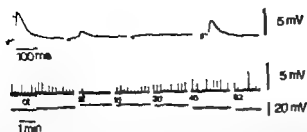


Fig. 5 ACh-potentials from muscle end-plate showing the effect of Triton X 100 (160×10^{-4} M). The iontophoretic pulses (4 ms duration, 5×10^{-4} A) are delivered every 14 ms and the ACh-potentials recorded (upper and middle traces). Prior to detergent application curare (0.5×10^{-4} M) was applied (not shown) and the fibre allowed to recover about 45 min. At the first arrow Triton X 100 was added to the Ringer solution and after about 5 min (second arrow) the fibre was returned to normal Ringer solution. The record is later speeded at times indicated by numbers below the middle trace. At 6 min the response has decreased to about 60% of control amplitude. Insets above the middle trace show ACh-potentials at different times in an extended time scale. As is seen the time course of the response is little changed. The lower trace shows the change in resting membrane potential during the experiment. At the end of the experiment the intracellular electrode was withdrawn from the fibre in order to check the E_{Cl} (-92 mV). This particular fibre was small (a p.p.) and was probably a large fibre. The upper and the middle traces are recordings through an AC signal amplifier (bandwidth 0.1–3 000 Hz).

Methods. Before application of the detergent, the ACh-potentials were recorded during several changes of normal Ringer solution. If stable measurements were obtained, curare (δ -tubocurarine) was applied at concentrations varying from about 10^{-4} to 10^{-3} M and the effect and the subsequent recovery was followed. If the ACh-potentials returned to about the precurare amplitude the detergent was applied. In other cases, where the amplitude was considerably reduced or the time course of the ACh-potentials was grossly affected the fibre was discarded. In Fig. 5 is shown responses to iontophoretically applied acetylcholine from an end-plate of the cutaneous pectoris muscle before, during and after application of Triton X-100 (160×10^{-4} M). The iontophoretic electrode was placed about 100 μ m from the recording electrode and the pulses had a duration of 4 ms and a current of about 5×10^{-4} A. The decline in amplitude follows an exponential time course, $\tau_{\text{dec}} = 39$ s, see Fig. 6) which is more slow than seen after exposure to curare. Although no quantitative comparison was made the time course seen in the iontophoretic experiments seems to be in accordance with that found in the m.e.p.p. experiments (cf. Fig. 1 and 3). After return to normal Ringer solution (second arrow 5 min) the response slowly returned and after 60 min it was about 60% of control value. As shown in the top trace where representative ACh-potentials (at different times) are illustrated in an extended time scale, the time course of the ACh-potentials is approximately the same. In this end-plate the m.e.p.p.'s were small indicating a large fibre. The time needed for recovery is consistent with that as found for m.e.p.p.'s and in the same range found for the recovery of the maximum rate of rise of the action potential in frog muscle. The incomplete recovery can be accounted for if it is assumed that the effect is in part irreversible but it is equally possible that the iontophoretic electrode had been moved or that the fibre was deteriorating. This latter explanation is not probable because the resting membrane potential was found to be -92 mV at withdrawal of the recording microelectrode at the end of the experiment.

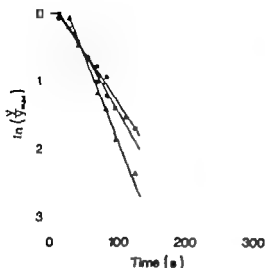


Fig. 6. The time course of the decrease in amplitude of the ACh-response after application of TX-100 (160×10^{-6} M) for three different end-plates. Abscissa time in sec after application of Triton X 100. Ordinate $\ln(y/y_{\max})$ where y is the amplitude of ACh-response at time t and y_{\max} is the amplitude before Triton X 100 was applied. The times for onset are somewhat arbitrarily chosen because of the ill defined change of solution (see methods). Individual τ_{on} values are 42 s, 59 s and 67 s. τ_{off} estimated from the relation $\ln(y/y_{\max}) = -(\tau_{\text{off}}/t)$. This presupposes that the ACh-response is completely abolished which was not the case for one fibre (●) (see text).

Another explanation is that the current through the ACh pipette was reduced but this is probably not the case the resistance of the pipette was only little changed as measured before and after the experiment. It is thus obvious that Triton X 100 can alter the response of the membrane potential produced by acetylcholine. This provides evidence that Triton X 100 exerts its action on the cholinergic receptor of the postsynaptic membrane.

Kinetic analysis

An estimation of the dissociation constant for Triton X 100 was performed (see Warr 1968) using three muscles in which the time courses for the onset of the effect were obtained. In Fig. 6 is plotted in a log diagram the reduction in the amplitude of the ACh-potential versus time after 160×10^{-6} M Triton X 100 was applied. In two fibres the time course is initially exponential, while in the third (●) there is a considerable deviation from an exponential. In this fibre the response was, however, not completely abolished but seemed to reach a steady state value of about 16% of the control value. After subtraction of this steady state value the curve was replotted (not shown) an exponential time course was then obtained. The mean value from these fibres for the time constant of onset (τ_{on}) is 56 s and using the actual concentration 160×10^{-6} M the forward (association) rate constant (k_1) is $1.1 \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$. This calculation of k_1 presupposes that the time constant for onset (τ_{on}) is much shorter than that for offset (τ_{off}) which seems to be justified in the present study (Fulpius 1976). The time constant for offset (τ_{off}) was more difficult to estimate because of the virtually irreversible effect of Triton X 100. A rough approximation of 30 min was chosen on basis of several experiments with iontophoretic application (cf. Fig. 8). This gives a value of the dissociation rate constant (k_2) of $5.6 \times 10^{-4} \text{ s}^{-1}$. The dissociation constant ($K_D = (k_2/k_1)$) thus arrived at is $5 \times 10^{-8} \text{ M}$. This value should be compared to the K_D -values for Triton X 100 of the maximum rate of rise of the muscle action potential which was found to be $35 \times 10^{-8} \text{ M}$ using steady state kinetics (Rydqvist 1977). The K_D value for the end plate is probably too low as judged from the very small effect by for example, $40 \times 10^{-8} \text{ M}$ and it is probable that the reaction is not of a first order (R_1)

which will be discussed later. In order to estimate the accuracy of the method the dissociation constant for tubocurarine was calculated using results from three experiments. The values found were $k = 4.1 \cdot 10^4 \text{ s}^{-1} \text{ M}$ (range $2.5\text{--}5.2 \cdot 10^4 \text{ s}^{-1} \text{ M}^{-1}$) and $k = 1.8 \cdot 10^4 \text{ s}^{-1}$ (range $1.0\text{--}2.7 \cdot 10^4 \text{ s}^{-1} \text{ M}^{-1}$) which give a dissociation constant $K = 4 \cdot 10^{-6} \text{ M}$. This is in reasonable agreement with values found by other authors (van Maanen 1930, Jenkinson 1950, Highman *et al.* 1963). The method is thus reliable for estimating the kinetic parameters for Triton but the values should nevertheless be regarded as approximate.

Structural observations

The electrophysiological findings described above suggest both a presynaptic and a postsynaptic effect produced by the detergent. In order to investigate if morphological changes might accompany these effects a series of muscle preparations soaked for 30 min in Triton X 100 ($160 \cdot 10^{-6} \text{ M}$) were prepared for electronmicroscopic observation on freeze-fracture replicas as well as in longitudinal ultrathin section.

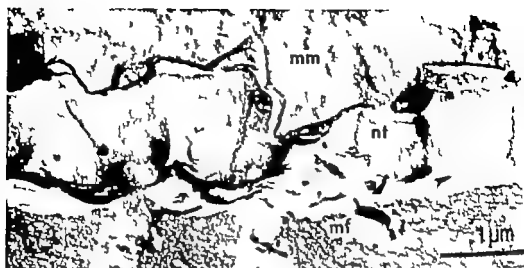
Freeze-fracture replicas. About 10 pairs of cutaneous pectoris muscles were included in a freeze-fracture study, one muscle being soaked in Triton X 100 ($160 \cdot 10^{-6} \text{ M}$) for 30 min, the twin muscle being used as control. The untreated muscle end-plate had the same normal appearance as described by Heuser *et al.* (1974) and by Peper *et al.* (1974). In Fig. 7 is illustrated a replica of a typical normal end-plate, the nerve terminal (nt) being viewed from the outside (PF-side, Branton *et al.* 1976). In the upper part of the picture is seen the muscle membrane (mm), PF-side, and in the lower part the interior of the muscle (mf). The terminal is characterized by the elevated ridges traversing the terminal (see also Fig. 9) which on both sides are lined with an array of particles arranged as a double row. The particles have a diameter of approximately 10 nm. In ultrathin sections these ridges are seen as thickenings of the presynaptic membrane described as active zones (Jensen *et al.* 1970). The terminal shown in Fig. 7 had also an abundance of smaller particles spread over the terminal membrane. These particles were not always present in normal control end-plates. Such abundance of particles is thought to indicate an active end-plate (Heuser 1976) but in the present study where virtually all preparations were using end-plates, this functional correlation was not obvious.

Fig. 7 Freeze-fracture view of control nerve terminal from the cutaneous pectoris muscle of the frog fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.075 M Na-cacodylate buffer. The nerve terminal (nt) is viewed from the outside (PF-side). On the upper part of the picture is seen the muscle membrane (mm) (PF-side) and in the lower part is seen the interior of the muscle fibre (mf). The presynaptic ridges of the nerve terminal are prominent, lined with a double row of particles (10 nm) on each side. In this terminal particles are seen in abundance between ridges. No doublets are seen in the vicinity of the ridges which indicate resting nerve terminal. Muscle membrane (mm), nerve terminal (nt), interior of muscle fibre (mf).

Fig. 8 Freeze-fracture view of nerve terminal which have been soaked in Triton X-100 ($160 \cdot 10^{-6} \text{ M}$) for 30 min and subsequently fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.075 M Na-cacodylate buffer. The view is the same as in Fig. 7. In this terminal several broken away areas of the nerve membrane are seen (asterisks). At some distance away from the ridges are seen particles of the same size (arrows) as those lining the ridges. The end-plate was very large and fewer particles are seen between the ridges. Twin muscle of that in Fig. 7.

Fig. 9 High magnification of presynaptic ridge from the control terminal seen in Fig. 7.

Fig. 10 High magnification of the Triton exposed terminal membrane seen in Fig. 8. Note clusters of particles away from the ridge (arrows) and broken away membrane patches (asterisks). The particles (arrows) are of the same size as those found lining the ridge (about 10 nm).



replica of the Triton treated twin muscle nerve terminal (Fig. 8 and 10) revealed a more varied general appearance with several irregular distortions (asterisk) which are probably sites where the membrane is broken away displaying the interior of the terminal or the space place through the membrane might be altered. These distortions were also occasionally seen in the muscle membrane. The ridges (Fig. 8) are seen somewhat more irregular particles lining the ridges are not arranged in the strict manner seen in normal end-plates. In addition clusters of particles (10 nm) are seen at some distance away from the ridges (arrows) Fig. 10 is an enlarged portion of an area which shows both the extra particles and the above mentioned distortions. Particle aggregations like these were occasionally seen in end-plates soaked in the detergent but never in control muscles. The postsynaptic muscle membrane, which is characterized by an abundance of intramembraneous particles, thought to be the acetylcholine receptor protein and mostly seen on the cytoplasmic (PF) side of the membrane was observed in several replicas of both normal and detergent treated muscles, but no obvious difference in the number or arrangement of these particles could be seen. In summary it then seems that treatment with Triton X 100 induces small or no changes in the postsynaptic membrane, whereas in the synaptic membrane particles of the same size as those lining the ridges appear at some distance away from the ridges, as if lateral diffusion of the particles had occurred.

Materials and methods

Out 10 pairs of cutaneous pectoris muscles were used, one muscle soaked in Triton X 100 (160×10^{-4} M) for 30 min, the twin muscle being used as control. After fixation the muscle fibres were embedded for longitudinal sectioning. In most cases 1 μ m sections, stained with toluidine blue, were first examined in the light microscope to search for end-plate regions, and ultrathin sections were subsequently obtained.

In control muscles the neuromuscular junction had the same general appearance as described by Birks *et al.* (1960) and by Dreyer *et al.* (1973). However using tannic acid as a fixative usually gave improved preservations of double membranes and in particular the synaptic membrane was densely stained (Fig. 11 and 15). In high magnification this membrane was well over 10 nm at the upper portion of the junctional fold, but transformed to a considerably thinner membrane seen to outline the base of the fold (Fig. 11 and 15). This is in accordance with earlier findings using horseradish peroxidase-labelled alpha-bungar-

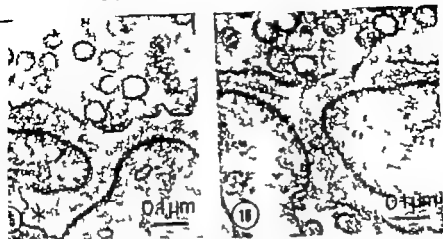
Fig. 11. Longitudinal section of control end-plate from frog cutaneous pectoris muscle soaked in normal saline solution for 30 min after dissection. This muscle was fixed in 3% glutaraldehyde and 3% paraformaldehyde in 0.05 M Na-cacodylate with 2 mM CaCl_2 added. Mitochondria (m), synaptic vesicles (sv), nerve zone (nz), junctional fold (f).

Fig. 12. Longitudinal section of an end-plate from the twin cutaneous pectoris muscle of that in Fig. 11 soaked in 160×10^{-4} M Triton X 100 for 30 min after dissection. Fixation procedure the same as described in Fig. 11. Nerve fibre mitochondria (m) are less dense than in the control. Note well defined matrix of single mitochondria.

Fig. 13. Detail of control nerve terminal of Fig. 11 showing mitochondria and synaptic vesicles.

Fig. 14. Detail of detergent nerve terminal from the same end-plate as in Fig. 12. Triton X-100 (160×10^{-4} M) was used. The mitochondria are swollen with small internal cristae. Synaptic vesicles are of the same diameter as in control (Fig. 13).





15 Detail of synaptic junction from the control end-plate in Fig. 11. Note the increased thickening of postsynaptic membrane on the upper part of the junctional fold (arrow) as compared to the thin base at the base of the junctional fold (asterisk).

16. Equivalent region as in Fig. 15 from Triton exposed end-plate of Fig. 12. The membranes have exactly the same appearance as in the control picture (Fig. 15).

acid (Fertuch *et al.* 1974; Daniels *et al.* 1975; Lentz *et al.* 1977). This indicates that acetic acid might have a favourable effect on the staining of the postsynaptic membrane which is rich in acetylcholine receptors. In the nerve terminal, vesicles were clearly outlined, the active zones (Couraux *et al.* 1970) seen as densely stained areas in the presynaptic membrane over a junctional fold (Fig. 11 and 15). Schwann cell processes are occasionally seen intervening the active zones (*cf.* freeze-fracture replicas). The mitochondria inside the nerve terminal were found to be irregular in shape with somewhat obscured cristae in contrast to the mostly well defined cristae in the muscle cells. After the muscle was soaked in 10^{-4} M Triton X 100, prior to fixation, changes in the general appearance of the end-plate regions were minor. However the mitochondria of the nerve terminal were noticeably more swollen than in control preparations. This was consistently seen in all fiber concentrations used, the general appearance of the mitochondria differing somewhat (Fig. 11 and 12, Fig. 13 and 14). The mitochondria of the muscle cell were as a rule better curved (Fig. 11), at least those found in the vicinity of the end-plate. The reason for this is not clear but a diffusion barrier due to the covering of the nerve terminal and its Schwann cell might be one cause. A close examination of the pre- and postsynaptic membrane at higher magnification revealed no obvious difference between treated and untreated muscles (Fig. 15 and 16).

Discussion

The results obtained in the present study show that Triton X-45 as well as Triton X 100 block the neuromuscular transmission but leave the resting membrane potential and membrane resistance relatively unaffected. This block is of postsynaptic origin and is revealed by a decrease in m.e.p.p. amplitude and a decrease in amplitude of iontophoretically

induced ACh-potentials. In addition there is a presynaptic effect, seen as an increase in m.e.p.p. frequency after application of Triton detergents.

Presynaptic effects In the electron microscope the mitochondria in the presynaptic terminal are noticeably swollen after treatment with Triton X 100 (Fig. 12 and 14). Leakage of Ca^{2+} from damaged mitochondria may thus be the cause of the increase in m.e.p.p. frequency. The notion that the effect on m.e.p.p. is influenced by mitochondrial membrane activity is supported by the finding that there is a large increase in frequency following the addition of dicoumarol, an uncoupler of oxidative phosphorylation (Alnaes *et al.* 1975). Dicoumarol is thought to reduce the active uptake of Ca^{2+} which is known to occur (Lehninger 1970) and will lead to an increase in intracellular Ca^{2+} concentration. Another compound, ruthenium red, which specifically inhibits Ca^{2+} by mitochondria (Moore 1971; Vasington *et al.* 1972) also increases the m.e.p.p. (Rahaminoff *et al.* 1973). Alternative mechanisms should also be considered. For example, increased Ca^{2+} entry from the extracellular space could give the same effect. It is known that increased Ca^{2+} concentration in the external medium increases the m.e.p.p. frequency (Boyd *et al.* 1956; Hubbard 1961; Mambrini *et al.* 1964; Matthews *et al.* 1977).

Another possible action of the detergents is that by their association with the lipid part of the nerve terminal they increase the fluidity of the membrane, i.e., the freedom of motion of the phospholipid molecules might increase, thereby increasing the probability of fusion between the vesicles and the nerve membrane (Papahadjopoulos *et al.* 1975a, b; Pagano *et al.* 1975; Van der Bosch *et al.* 1975). Support for this idea is provided by the freeze-fracture observations seen in Fig. 8 and 10 where the nerve membrane is covered with a great number of broken away patches, and particles of the same apparent size as those lining the ridges arranged in clusters at some distant way from the ridges. One explanation for these extraridge clusters might be a lateral diffusion of particles from the ridge due to altered physical properties of the lipid phase.

Postsynaptic effects By iontophoretic application of ACh during exposure to Triton X 100 it was possible to follow the effect on the postsynaptic membrane. In three cells it was possible to study the recovery and to get an estimate of the time constant for onset and offset of the reaction. The time constant for onset (τ_{on}) of the reaction for the three cells was $\tau_{\text{on}} = 56$ s, while the time constant for offset (τ_{off}) of the reaction was estimated to be in the order of 30 min ($\tau_{\text{off}} = 1800$ s) (cf. Fig. 5). According to these data the dissociation constant (K_D) for the reaction between Triton X 100 and some postsynaptic site was found to be 5×10^{-4} M as calculated from the quotient between the dissociation rate constant and the association rate constant (k_1). The K_D value for the effect of Triton X 100 should be regarded with caution because the calculation of this value presupposes that the reaction is of first order, i.e., one molecule of Triton X 100 reacts with one site on the receptor structure. This is probably not true although no quantitative measurements were made of the dose-response relation. Looking at the blocking effect of e.g. 40 , 80 and 160×10^{-4} M it was obvious that the dose-response curve was rather steep, i.e., the Hill coefficient probably being bigger than one. The effect of 40×10^{-4} M takes very long time to develop and a plausible concentration at which Triton X 100 exerts 50% effect on the ACh-potential must be well over 40×10^{-4} M. This does not fit with the K_D value found (5×10^{-4} M).

is assumed that two Triton X-100 molecules simultaneously have to bind to the receptor structure a value for 50% effect of $53 \cdot 10^{-6}$ M is obtained which fits better with approximate dose-response relation.

The effect on the postsynaptic membrane is interpreted as an effect on the acetylcholine receptor resulting in an inhibition of the permeability increase, normally following the action between ACh and the receptor. One possibility is that Triton X 100 has affinity to the same sites as ACh, e.g. a competitive antagonist, or to other sites on the receptor protein. This cannot be settled without measurements of ACh responses as a function of ACh output from the iontophoretic pipette, after application of different concentrations of Triton X 100. According to the moderate affinity of the detergent to the postsynaptic site action on the stereospecific ACh sites, like curare, seems less probable. An alternative is that the detergent has affinity to other sites which either may mask the ACh sites or modify conformational changes that are assumed to underlie the ionophore activity of the receptor protein and thus inhibit the permeability increase for sodium and potassium

(Monod *et al.* 1965). It has been shown (Brismar *et al.* 1975) that Triton X 100 decrease response to carbamylcholine at the endplate and inhibit the binding of H-nicotine to the partially purified receptor protein, in contrast to sodium-dodecyl-sulphate (SDS) another type of detergent which reduces the response to carbamylcholine but does not inhibit nicotine binding. This together with the present findings that the resting membrane potential and the membrane resistance are only little affected give no support for a mechanism where Triton X 100 interacts with the lipid phase changing it by hydrophobic interactions (Sjoklovicz 1975 Rydqvist 1977) and thereby altering the function of the acetylcholine receptor. However the ultrastructural observations in freeze-fracture replicas and ultrathin sections show no changes in the postsynaptic membrane which are relevant for the electrophysiological results even though the resolution, in ultrathin sections, of these membranes is very good using tannic acid as a mordant.

The reversibility of the effect of the detergent is in some doubt. In most cases it was not possible to obtain the control response after the preparation had been returned to normal Ringer solution. As mentioned before this could be explained by the considerable time constant for offset of the reaction, and a general rundown of the fibre which might affect response. Also, the resistance of the iontophoretic micropipette might have been altered during the recording. It was repeatedly experienced that after a recording from a fibre over a period of more than 1 h, impaling another fibre of the same muscle gave perfectly normal responses including m.e.p.p.s indicating that the action was reversible. Another explanation is that an irreversible component is present (Brismar and Rydqvist 1978). Close inspection of Fig. 6 reveals that more than one time constant seem to be involved in the onset of the reaction between Triton X 100 and the frog end-plate.

In summary both Triton X-45 and Triton X 100 are equally potent in blocking the neuromuscular transmission by an effect on the postsynaptic membrane. The results are more easily interpreted in terms of detergent-protein interactions than in terms of detergent-lipid interactions, i.e. the detergent most likely has a *per se* action on the acetylcholine receptor. The effect is compatible with a drug-receptor interaction with an ED_{50} in the range of 5 to $50 \cdot 10^{-6}$ M for Triton X-100, the value being dependent on the type of reaction (Hill

coefficient probably larger than one) The site on the receptor protein to which detergents have affinity has not been possible to determine with the present technique.

The detergents also have a presynaptic effect, the m.e.p.p frequency being increased probably due to raised Ca^{++} concentration inside the nerve terminal This increase in intracellular Ca^{++} is most likely due to a perturbation of the mitochondrial membrane the detergent these membranes perhaps being more susceptible to the action of Td detergents than the nerve plasma membrane.

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Influence of intravenously administered catecholamines on cerebral oxygen consumption and blood flow in the rat

By

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Abstract

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order to study effects of catecholamines on cerebral oxygen consumption (CMR_{O_2}) and blood flow (CBF), rats anesthetized on 75% N_2O and 25% O_2 were infused i.v. with noradrenaline (2, 5, or 8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or adrenaline (2 or 8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 10 min before CBF and CMR_{O_2} were measured. In about 30% animals infused with 2-8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ of noradrenaline, CMR_{O_2} (and CBF) rose. However, there was dose-dependent response, and CMR_{O_2} did not exceed 130% of control. The effects of noradrenaline in rats of 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ on CMR_{O_2} and CBF were blocked by propranolol (2.5 $mg \cdot kg^{-1}$). In animals infused with adrenaline (8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) CMR_{O_2} was doubled and, in many, CBF rose 4- to 6-fold. It is concluded that, in sufficient amounts, catecholamines have pronounced effects on cerebral metabolism and blood flow: the effects of adrenaline on CMR_{O_2} and CBF resembling those observed in status epilepticus.

Key words: CBF, CMR_{O_2} , adrenaline, noradrenaline, propranolol.

Results reported from this laboratory have shown that, at least in some rat strains, three conditions are associated with an increase in cerebral oxygen consumption (CMR_{O_2}) to about 200% of control, and with a marked rise in cerebral blood flow (CBF). These conditions, which are unaccompanied by seizure patterns in the EEG include (1) withdrawal of stroke occlude supply in paralyzed animals in which pain and discomfort were minimized (Carlsson *et al.* 1975-1977), (2) amphetamine administration (Bernthman *et al.* 1976, 1978), and (3) arterial hypoxia (Bernthman *et al.* 1977; Bernthman *et al.*, submitted for publication). In the first of these conditions ("immobilization stress"), the increases in CMR_{O_2} and CBF were prevented by adrenalectomy or by prior administration of propranolol, suggesting that circulating catecholamines were involved (Carlsson *et al.* 1977). This suggestion was supported by results showing that the increase in CMR_{O_2} during hypoxia was considerably reduced by adrenalectomy (Bernthman *et al.* 1977).

The objective of the present study was to find out whether systemically administered

catecholamines can increase CMR_{O_2} and CBF to values measured in these three "control" situations. To that end, paralyzed rats maintained on 70% N_2O were infused with varying doses of noradrenaline and adrenaline. Cortical CBF was measured with a technique based on the Fick principle, and CMR_{O_2} was calculated from CBF and venous differences in oxygen content.

Methods

Operative and sampling techniques

Male Wistar rats of a B.P.P. strain (Møllegaard Avslaboratorium, Copenhagen) were anesthetized with 2.3% halothane. When unresponsive the animals were tracheotomized and connected to a Stryker respirator delivering gas mixture at a rate that gave arterial CO_2 tensions of 35–40 mmHg. All rats were ventilated with 75% N_2O and 25% O_2 . In some 0.6–0.8% halothane was added to the gas mixture during operative procedures but, as soon as these were completed, ventilation was continued on N_2O – O_2 mixture. Body temperature was kept close to 37°C by heating pads.

Both femoral arteries were cannulated to allow blood pressure recording and sampling of arterial blood for measurements of blood gases, pH, and ^{133}Xe activity during CBF measurements. One femoral vein was cannulated for infusions of drugs, and the other for infusion of donor blood during rapid sampling of blood for CBF measurements. The caudal portion of the superior sagittal sinus was exposed by a burr hole for sampling of (cortical) cerebrovenous blood.

Infusion of catecholamines

Adrenaline and noradrenaline were obtained as the bitartrates, dissolved in physiological saline at a concentration of 1 mg ml⁻¹ (ACO, Stockholm). Before infusion, 0.18, 0.50 or 0.80 ml of the solution were mixed with physiological saline to a final volume of 10 ml yielding amine concentrations of 18, 50 and 80 µg ml⁻¹ respectively. About 30 ml following the completion of operative procedures adrenaline and noradrenaline solutions were infused at rates of 2, 5 or 8 µg kg⁻¹ min⁻¹. The volumes of solution infused were less than 0.5 ml. Some animals were given propranolol (Indinor) 2.5 mg kg⁻¹ 30 min before CBF measurement. Saturation with ^{133}Xe was started 10 min before the infusion of catecholamines began. Desaturation was started when the catecholamines had been infused for 10 min. Thus, CBF and CMR_{O_2} values obtained pertain to a total infusion period of 10–15 min.

Analytical techniques

Arterial P_{O_2} , P_{CO_2} and pH were measured at 37°C, using microelectrodes of the Beckman (Klett) Radiometer (Copenhagen) types. All values were corrected for temperature. Arterial and cerebrovenous oxygen content (Co_2) were measured polarographically on 25 µl samples (Fabel and Løbbens 1964, Berntman *et al.* 1974).

Cerebral (cortical) blood flow was measured with ^{133}Xe modification of Kety and Schmidt (1948) technique, using a partition coefficient for Xenon of 0.83. The basic methodology was as described by Norberg and Sjö (1974), with the modifications introduced by Berntman *et al.* (submitted for publication). When desaturation was started, and arterial and cerebrovenous samples were collected by rapid succession, donor blood was slowly infused to maintain blood pressure. Total amount of blood infused was 3–4 ml. Since the CBF (and CMR_{O_2}) obtained is determined mainly by the first 90–120 s of desaturation, the relevant amount infused did not exceed 2 ml. CMR_{O_2} was calculated by multiplying CBF with the arteriovenous difference in oxygen content (AVD_{O_2}). The latter was determined in at least triplicate: the first set of samples being taken just prior to desaturation and the last 2–3 min later. If AVD_{O_2} differed by more than 10% between two consecutive samples, the experiment was discarded. If not, AVD_{O_2} was used to calculate CMR_{O_2} .

Statistical differences were calculated using Student's *t* test. The following symbols are used: $p < 0.05$, $p < 0.01$, $p < 0.001$.

Results

Physiological variables are shown in Table I. Values for body temperature P_{O_2} , P_{CO_2} and pH were obtained just after CBF measurements. Mean arterial blood pressure was con-

TABLE 1. Body temperature and mean arterial blood pressure (MABP), as well as arterial P_{aO_2} , P_{aCO_2} , and pH in control rats, and in those infused *i.v.* with various doses of noradrenaline and adrenaline for 10–15 min.

	Infusion rate $\mu\text{g kg}^{-1} \text{ min}^{-1}$	Temp. $^{\circ}\text{C}$	MABP (mmHg)			P_{aO_2} mmHg	P_{aCO_2} mmHg	pH
			Preinfus.	Max.	Flow			
control	—	15	37.0 ± 0.1	151 ± 4	140 ± 3	125 ± 5	38.0 ± 0.5	7.375 ± 0.014
adrenaline	2	6	37.3 ± 0.3	149 ± 6	178 ± 6	143 ± 3	105 ± 5	37.5 ± 1.2
	5	8	37.8 ± 0.4	149 ± 5	178 ± 8	140 ± 3	108 ± 5	43.8 ± 2.7
	8	7	37.2 ± 0.2	156 ± 7	181 ± 3	149 ± 5	101 ± 5	37.7 ± 1.3
	—	—	—	—	—	—	—	—
noradrenaline	2	5	37.5 ± 0.2	146 ± 3	167 ± 3	141 ± 5	011 ± 4	38.2 ± 1.3
	5	8	37.0 ± 0.2	149 ± 6	180 ± 6	159 ± 3	117 ± 5	39.3 ± 2.5
	—	—	—	—	—	—	—	—

Values are means \pm S.E.M. Control values were taken from Bernstein *et al.* (submitted for publication).
0.05, $p < 0.01$ $p < 0.001$

usually recorded. Values given are those recorded before catecholamines were infused, peak pressures obtained during infusion, and values obtained at the beginning of desaturation.

Some animals infused with noradrenaline had moderately elevated temperatures and increased CO_2 tensions. Thus, with an infusion rate of $5 \mu\text{g kg}^{-1} \text{ min}^{-1}$ three animals had arterial CO_2 tensions of 50, 51 and 53 mmHg, respectively. Peak MABP was close to 180 mmHg but, during desaturation, values were close to normal. There was a moderate plasma sodium. Animals infused with $5 \mu\text{g kg}^{-1} \text{ min}^{-1}$ after administration of propranolol (5 mg kg^{-1}) showed similar changes in physiological variables. Arterial P_{aO_2} was close to or above 100 mmHg in all groups. Arterial oxygen content was similar in all groups (about $9 \mu\text{mol ml}^{-1}$). Venous oxygen content was decreased in propranolol-treated animals (from a control value of 6.07 ± 0.23 to $4.69 \pm 0.43 \mu\text{mol ml}^{-1}$).

Animals infused with adrenaline in dose of $2 \mu\text{g kg}^{-1} \text{ min}^{-1}$ showed values similar to those found in animals infused with noradrenaline. With an infusion rate of $8 \mu\text{g kg}^{-1} \text{ min}^{-1}$ MABP was elevated at the time of desaturation. Two animals had increased CO_2 tensions (50 and 52 mmHg, respectively). Mean venous oxygen content was significantly increased (from a control value of 6.07 ± 0.23 to $7.28 \pm 0.44 \mu\text{mol ml}^{-1}$).

Fig. 1 shows individual CBF and CMR_{O_2} values obtained during infusion of noradrenaline. With $2 \mu\text{g kg}^{-1} \text{ min}^{-1}$ three CMR_{O_2} values of six were increased above control and, after $5 \mu\text{g kg}^{-1} \text{ min}^{-1}$ CMR_{O_2} rose in at least five of eight animals. Results obtained with an infusion rate of $8 \mu\text{g kg}^{-1} \text{ min}^{-1}$ indicate that CMR_{O_2} did not increase in proportion to dose ("all or none response"). There was considerable scatter in CBF values. Marked increases were observed only in the three hypercapnic animals and, in many the increase in CBF was small or moderate.

Fig. 2 shows that, in propranolol-treated animals, infusion of noradrenaline ($5 \mu\text{g kg}^{-1}$

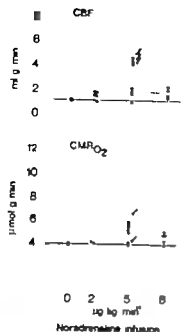


Fig. 1

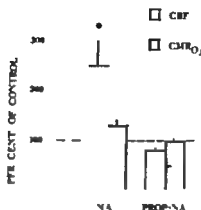


Fig. 2

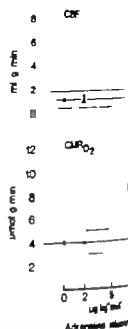


Fig. 3

Fig. 1 Individual $CMRO_2$ and CBF values measured in animals infused with various doses of noradrenaline. Mean control values are given by large filled circles, and ± 2 S.D. by dotted lines. Values obtained in hypercapnic animals (P_{aCO_2} 50–53 mmHg) are denoted by arrows. Note absence of dose-dependent response.

Fig. 2 Influence of propranolol (2.5 mg kg^{-1} given 30 min prior to CBF measurements) on changes CBF and $CMRO_2$ (in percent of control) following infusion of noradrenaline ($5 \text{ µg kg}^{-1} \text{ min}^{-1}$). Values obtained without propranolol during noradrenaline (NA) infusion are given to the left, those obtained in propranolol-treated animals to the right. (Prop+NA). $p < 0.01$

Fig. 3 Individual CBF and $CMRO_2$ values measured in animals infused with 2 or $8 \text{ µg kg}^{-1} \text{ min}^{-1}$ adrenaline. Mean control values are given by large filled circles, ± 2 S.D. by dotted lines. Values obtained in hypercapnic animals (P_{aCO_2} 50–52 mmHg) are denoted by arrows.

min^{-1}) was unaccompanied by increases in $CMRO_2$ and CBF. In fact, there was a slight decrease in CBF.

Infusion of adrenaline had effects different from those observed after noradrenaline (Fig. 3). With $2 \text{ µg kg}^{-1} \text{ min}^{-1}$ only one $CMRO_2$ value was increased above control, while $8 \text{ µg kg}^{-1} \text{ min}^{-1}$ gave rise to an excessive rise in oxygen consumption. Thus, in six of eight animals $CMRO_2$ exceeded $8.2 \text{ µmol g}^{-1} \text{ min}^{-1}$ (200% of control) and only two had $CMRO_2$ values in the range observed with infusion of noradrenaline. In these two animals CBF was only moderately increased (1.7 and $2.5 \text{ ml g}^{-1} \text{ min}^{-1}$ respectively) but in the majority of the remaining animals, CBF increased 3- to 6-fold. In the latter group, two animals had CBF values of 5.7 and $7.3 \text{ ml g}^{-1} \text{ min}^{-1}$ in spite of the fact that arterial P_{CO_2} was lower than 40 mmHg.

In order to allow a comparison of increases in CBF and $CMRO_2$ during infusion of noradrenaline and adrenaline all individual CBF values were expressed as per cent of the value expected at their measured P_{CO_2} , assuming a 5% change in CBF per mmHg change in P_{CO_2} (Norberg and Sjö 1974). The results are shown in Fig. 4. Following noradrenaline infusion, there was a relatively poor correlation between increases in $CMRO_2$

CATECHOLAMINES AND BRAIN METABOLISM

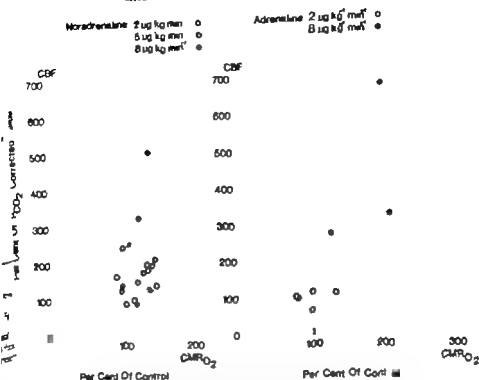


Fig. 4 Relationship between increases in CMR_{O_2} and CBF in subjects infused with noradrenaline and adrenaline. The values are expressed in percent of control values. In deriving percentage changes from control, CBF values were first corrected. Each individual CBF value is related to the control value, expected the actual PCO_2 assuming a 5% change in CBF per mmHg change in PCO_2 .

and CBF but, in the majority of animals, there was a proportionally larger rise in CBF than in CMR_{O_2} . This tendency was also observed in adrenaline-infused animals and, in two animals, CBF increased 6-fold for a 2-fold rise in CMR_{O_2} .

Discussion

In interpreting the present results, one difficulty arises from the fact that catecholamines are known to poorly penetrate the blood-brain-barrier (Well-Malmström *et al.* 1961; Bertler *et al.* 1966; Oldendorf 1971). Data published in the literature on cerebral metabolic and circulatory effects of systemically administered catecholamines are inconsistent. The first clear indication that catecholamines may affect cerebral metabolism and blood flow in man was reported by King *et al.* (1952) who found that large doses of adrenaline *iv* induced a 20% rise in CMR_{O_2} and CBF whereas noradrenaline in similar doses only caused a small reduction in flow. A subsequent study of equally large doses of adrenaline (given *iv*) failed to corroborate the effects on CMR_{O_2} and CBF (Semenbach *et al.* 1953). However like King *et al.* (1952) these authors noted that noradrenaline gave rise to a reduction in CBF and several other groups have obtained comparable results (e.g. Greenfield and

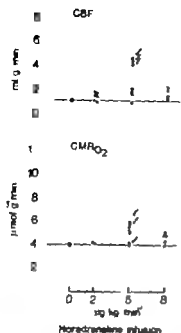


Fig. 1

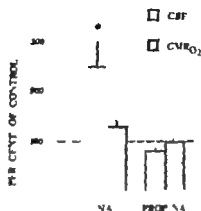


Fig. 2

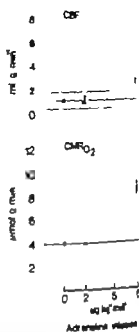


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Fig. 2 Influence of propranolol (2.5 mg kg^{-1} given *Le* 30 min prior to CBF measurements) on changes in CBF and $CMRO_2$ (in percent of control) following infusion of noradrenaline ($5 \text{ µg kg}^{-1} \text{ min}^{-1}$). Values obtained without propranolol during noradrenaline (NA) infusion are given to the left, those obtained in propranolol-treated animals to the right. (Prop+NA). $p < 0.01$

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In order to allow a comparison of increases in CBF and $CMRO_2$ during infusion of noradrenaline and adrenaline all individual CBF values were expressed as per cent of the value, expected at their measured P_{CO_2} , assuming a 5% change in CBF per mmHg change in P_{CO_2} (Norberg and Sjöjög 1974). The results are shown in Fig. 4. Following noradrenaline infusion, there was a relatively poor correlation between increases in $CMRO_2$

Results obtained on CBF following administration of noradrenaline and adrenaline are compatible with the presence of a constrictory α -adrenergic effect (see Ekström-Jodal *et al.* 1974, McDonnell *et al.* 1975), which is variably superimposed on the vasodilatory effect, the latter possibly being coupled to an adrenergic effect on metabolism. If it is assumed that adrenaline has equally strong effects on α - and β -receptors while noradrenaline dominating α -effects (and a weak β influence), it is possible to explain both the metabolic and the circulatory effects observed presently. It is of interest that in three animals infused with noradrenaline, moderate hypercapnia (P_{CO_2} 50–53 mmHg) was associated with marked rise in CBF. Possibly these results reflect the fact that hypercapnia blocks α -receptor activity without influencing β -receptor stimulation (see, however McDonnell *et al.* 1975).

The present results leave open the question how circulating catecholamines exert their effects on cerebral metabolism and blood flow. In view of present knowledge on blood-brain barrier permeability to catecholamines (see Introduction) a direct cerebral effect could either be due to penetration into areas with an imperfect barrier or to an "opening" of the tight barrier due to the increase in blood pressure. Results supporting the latter alternative have been published (MacKenzie *et al.* 1976, Hardebo *et al.* 1977). We are presently studying whether increases in CMR_{O_2} and CBF correlate with signs of blood-brain barrier dysfunction. However whatever is the mechanism, the present results show that catecholamines have pronounced effects on CMR_{O_2} and CBF and it is of considerable interest that adrenaline may increase CMR_{O_2} and CBF to values close to those observed during status epilepticus (see Meldrum and Nilsson 1978, Blennow *et al.* 1978).

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Tindall 1968, Ekström-Jodal *et al* 1974). In contrast, Olesen (1972) concluded that neither noradrenaline nor adrenaline, when given by intracarotid infusion in unanesthetized rats, had any significant effect on CBF.

Recent experimental results demonstrate that, at least under some circumstances, systemically administered catecholamines may significantly influence CMR_{O_2} and CBF. Results obtained in dogs by Xanatos and James (1972) and by McDonnell *et al* (1975) led to the conclusion that *in vivo* infusion of isoprenaline and noradrenaline increase CMR_{O_2} and CBF as a β -receptor mediated effect. This effect which was assumed to be elicited from chemoreceptors, seemed to oppose a direct vasoconstrictory action (of noradrenaline) as α receptor stimulation. The method used by the authors may not have been well suited for studies of CMR_{O_2} and since barbiturate anaesthesia was used, they leave open the question whether catecholamines can increase CBF and CMR_{O_2} above unanesthetized control values. Recent data on monkeys (MacKenzie *et al* 1976) clearly demonstrate that *in vivo* adrenaline may increase CMR_{O_2} and CBF. However such effects were obtained only when noradrenaline was infused intracarotidally following prior "osmotic opening" of the blood-brain barrier or if the amine was injected intraventricularly. The authors conclude that noradrenaline augments cerebral metabolic rate if it passes the blood brain barrier and that the increase in CBF was secondary to activation of metabolism.

As stated, the objective of the present experiments was to study whether intravenously administered catecholamines can increase CMR_{O_2} and CBF to values previously observed in situations that must be considered stressful. In interpreting the results it should be recalled that the anaesthesia used (70% N_2O) does not by itself reduce CMR_{O_2} below normal (Carlsson *et al* 1976) and that the CBF method used can be considered quantitative as it is based on the Fick principle. The results amply confirm those of previous investigators (McDonnell *et al* 1975, MacKenzie *et al* 1976) in showing that noradrenaline increases CMR_{O_2} and CBF and the percentage increases observed presently are not different from those reported by these authors. However our results demonstrate that, during noradrenaline infusion there was a considerable variability in response, and CMR_{O_2} did not rise above 150% of control. In contrast, when given in sufficient amounts, adrenaline provoked a doubling of CMR_{O_2} , with some CBF values reaching 500-600% of control. The increase in CMR_{O_2} following adrenaline infusion is similar to that previously observed in "immobility stress" in hypoxia, and following amphetamine administration (see Introduction), and the peak CBF values resemble those seen in animals given amphetamine. It is thus tempting to conclude that at least in the first two of these three situations, the changes observed in cerebral metabolic rate and blood flow were elicited by circulating adrenaline. So far, excessive changes have not been observed in all animal strains studied (see Bernthman *et al* 1977, 1978). By analogy we conclude that failure of observing a marked increase in CMR_{O_2} (or CBF) either was due to variations in release of adrenaline, or in its passage from blood to tissue. It should be recalled that this variability in response also has been observed in man (King *et al* 1952, Sensenbach *et al* 1975).

Our results confirm those of others (Xanatos and James 1972, McDonnell *et al* 1975, MacKenzie *et al* 1976) in showing that the influence of catecholamines on CMR_{O_2} (and CBF) is blocked by propranolol, suggesting that β -receptor stimulation is responsible.

Metabolic adaptation to hypoxia

Redox state of the cellular free NAD pools, phosphorylation state of the adenylate system and the (Na^+-K^+) -stimulated ATP-ase in rat liver

By

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Abstract

KIDONULA, V. L. and I. HASENDEM. *Metabolic adaptation to hypoxia. Redox state of the cellular free NAD pools, phosphorylation state of the adenylate system and the (Na^+-K^+) -stimulated ATP-ase in rat liver*. Acta physiol. scand. 1978. 104. 109-116.

The effect of hypoxia (30 mm 10% or 8% O_2) on the phosphorylation state and redox state of the cytosol and mitochondrial free NAD pools of rat liver were studied. Measurements were made both from normal animals and animals which had been exposed to the reduced partial pressure of oxygen (30.5 kPa or 40.8 kPa of air) for one or seven days. Cytosolic free NAD was reduced in the liver both in acute hypoxia and in hypoxia after one or seven days, i.e. the lactate/pyruvate and α -glycerol-3-phosphate/diacylglycerolphosphate ratios increased markedly. A marked reduction in the mitochondrial free NAD pool occurred only in acute hypoxia and only a slight reduction was observed in animals kept at 40.8 kPa for one or seven days, evaluated from the hepatic hydroxybutyrate/acetoacetate ratio. Liver ATP concentration decreased only in acute hypoxia without any significant recovery during one or seven days at 40.8 kPa. The hepatic ADP/P ratio decreased significantly with simultaneous decrease in the total adenine nucleotide concentration. A tendency was observed for the $ATP/ADP/P$ ratio to return to normal after seven days. The changes in acute hypoxia were significantly smaller than those noted in hypoxia after seven days, demonstrating an adaptation of the energy metabolism during prolonged hypoxia. Hepatic (Na^+-K^+) -stimulated ATP-ase activity was not affected by hypoxia.

Key words: Liver hypoxia, redox state of free NAD pool, phosphorylation state of the adenylate system, (Na^+-K^+) -ATP-ase, adaptation to hypoxia.

By measuring the ratio of the concentrations of oxidized and reduced substrates of a suitable, compartmentalized NAD-linked dehydrogenase, the redox state of the cytosol and mitochondria can be calculated separately (Bücher and Kilgus 1958). Acute ischemia of the liver achieved by clamping the hepatic artery and portal vein for 60 sec (Chance *et al.* 1965), and hypoxia of the animal (7% O_2 in the inhaled air) for 30 min (Lal and Miller 1973) result in an increase in the cytosolic NADH/NAD ratio and decrease in the ATP/ADP ratio of the liver (Chance *et al.* 1965). Anoxia results in a decrease in the hepatic ATP/ADP ratio, but the magnitude of the decrease and survival time in anoxia is dependent on the rate of ATP consumption (Ratna 1964; Mäenpää 1967).

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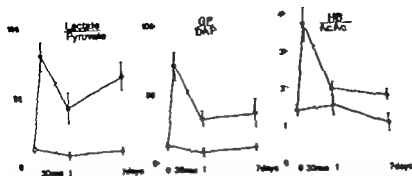


Fig. 1. Lactate/pyruvate, α -glycerol-3-phosphate/diacyclic phosphates and 3-hydroxybutyrate/acetoacetic ratios in the liver after various periods in moderate hypoxia. The animals were in the respirator 30 min under 10% O_2 . In the 1-day and 7-day experiments they were exposed to high altitude effects (5.5 kPa) for the given length of time before the 30 min period in the respirator under 10% O_2 . The results are means \pm S.E. from 4–10 separate experiments. Symbols: O, control; ●—● hypoxic and ■—■ pair; GP— α -glycerol-3-phosphate; DAP—diacyclic phosphates; HB, 3-hydroxybutyrate and AcAc, acetoacetic.

reproducible results, membrane fractions were isolated as follows: The homogenate was centrifuged 1 000 g for 10 min and the supernatant further centrifuged at 6 000 g for 15 min. This supernatant was centrifuged at 48 000 g for 30 min. The pellets from the 1 000 g and 48 000 g centrifugations were resuspended with the sucrose medium and diluted fourfold with water. The reaction was started by the addition of 0.2 ml of the homogenate, allowed to proceed for 4 min at 37°C, and stopped with trichloroacetic acid, and the inorganic phosphate (P_i) as determined as described by Ernster *et al.* (1950). The 4^{+} -ATP-stimulated ATPase activity was taken as the difference between the total ATPase activity and as observed in the presence of sodium and the absence of NH_4^+ and KCl . The values for the 1 000 g pellets were not reproducible, and therefore only the 48 000 g activities are reported here. The results are expressed as nmol of P_i /min per g wet weight of liver and nmol of P_i /min per mg protein. Protein was determined by the method of Lowry *et al.* (1951).

To test the validity of the method used, single intraperitoneal dose of L-thyroxine (6 μ g/kg in 10 mM NaOH-0.9% NaCl) was administered to rats, which were then killed 24 hours later. Within the NH_4^+ -stimulated ATPase activity in the 48 000 g sediment of the liver homogenate was 21.1 ± 7.5 (mean \pm S.D., 2) nmol P_i /min/mg protein, that in the thyroxine-treated animals was 34.2 ± 15.1 (mean \pm S.D., $n=2$).

Thus the method is sensitive enough to detect hormone-dependent changes.

Serum thyroxine determinations. Serum thyroxine (T_4) was determined by the modified method of Chopra (1972). A 20 μ l sample of serum was incubated overnight at 4°C with 100 μ l of antiserum in borate acid, BSA, 5-methyl-1-naphthalenephosphonic acid solution, pH 8.5, and 100 μ l (10 000 cpm) of [^{125}I]-thyroxine eluted (in borate acid, BSA solution, pH 8.5), the final medium containing 0.2 M borate acid pH 8.5, BSA 0.1 g/l and 5-methyl-1-naphthalenephosphonic acid 1.9 g/l. The immunocomplex was precipitated with polyvinylpyrrolidone (in 0.9% NaCl) to the final concentration of 12.5% (w/v) (Herscovici *et al.* 1974).

Statistical treatment. The two-tailed student's t-test for independent means was used for the statistical analysis, and P values of ≤ 0.05 were considered to indicate statistical significance.

Results

Lactate/pyruvate ratio. This ratio showed a significant increase in acute hypoxia (Fig. 1). After 30 min under 10% O_2 in the respirator the ratio increased by a factor of 6.0 ($p < 0.001$), and the ratio was elevated significantly by a factor of 3.2 ($p < 0.05$) after one day and 4.8 ($p < 0.001$) after seven days at 50.5 kPa following 30 min in the respirator under approximately the same partial pressure of oxygen (Fig. 1). The change in the lactate/pyruvate

Thyroxine administration increases the mitochondrial cytochromes, and certain enzymes of the oxidative metabolism (Kadenbach 1966). The hypermetabolic states of the rat resulting from thyroxine treatment, chronic ethanol administration or cold acclimation connected with increased $(\text{Na}^+ - \text{K}^+)\text{-stimulated ATP-ase activity}$ and the extra O_2 consumption noted under these conditions seems to be due to the increased ATP consumption by this enzyme (Ismael Beigi and Edelman 1971, Israel *et al.* 1973, Bernstein *et al.* 1971, also Israel *et al.* 1975). Hypoxia is a condition reported to result in decreased thyroid function (Galton 1972) and therefore changes in the $(\text{Na}^+ - \text{K}^+)\text{-stimulated ATP-ase}$ may be of significance in regulating ATP-turnover in low oxygen tension.

Material and methods

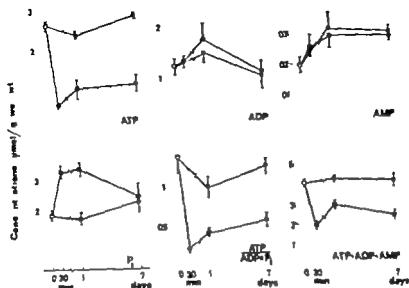
Reagents. Standard reagents were obtained from E. Merck AG Darmstadt, Germany. Lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, hydroxybutyrate dehydrogenase and the co-enzymes purchased from Boehringer GmbH, Mannheim, Germany and pyruvate kinase, glucose-6-phosphate dehydrogenase, hexokinase and myokinase from Sigma Chemicals, St. Louis, Mo. U.S.A. L-histidine and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A. $[\text{3-}^3\text{H}]\text{thyroxine}$ specific radioactivity 20–50 mCi/ μg , was obtained from the Radiochemical Centre, Amersham, Bucks, U.K., thyroxine antiserum from Farnos Ltd., Finland, 8-amino-1-naphthalene-sulphonic acid from Kodak Eastman Chemicals, Rochester, N.Y. U.S.A. and polyethyleneglycol from Fluka & Buchs, Switzerland.

Animals and hypoxic experiments. Sprague-Dawley rats from the Department's own colony were used. Both sexes were used and the ages of the animals varied between 3 to 4 months. Their food consisted of standard rodent chow (Astra-Ewos AB, Södertälje, Sweden). The animals were in each case divided into hypoxic, pair-fed control and *ad libitum* control groups.

For the metabolic determinations the rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally), the trachea was cannulated and respiration controlled with a rodent respiration pump (Harvard Apparatus Company Model 680). The respiratory rate was fixed at 60 cycles per min, and the tidal volume calculated from the equation $\log V = -1.57 + 0.735 \log m$, where m is the body weight of the animal in grammes and V the tidal volume in millilitres (Kleiman and Radford 1971). The control animals breathed room air for 30 min and the hypoxic animals $10\% \text{ O}_2$ or $8\% \text{ O}_2$ in nitrogen. In one group of hypoxic rats the animals were transferred to the respirator ($10\% \text{ O}_2$) for 30 min from a barometric pressure chamber in which they had been exposed to 50.5 kPa for one to seven days; these are referred to in the text as moderate hypoxia experiments. In another group of experiments the animals were taken to the respirator ($8\% \text{ O}_2$) from the barometric pressure chamber in which they had again been from one to seven days; these being referred to as severe hypoxia experiments. In studying $(\text{Na}^+ - \text{K}^+)\text{-stimulated ATP-ase}$ activity in hypoxia, only the hypobaric pressure chamber was used.

Metabolic determinations. At the end of the 30 min controlled ventilation in the respirator one lobe of the liver was freeze-clamped according to Wollenberger *et al.* (1960). The samples for the metabolic determinations were pulverized in a mortar under liquid nitrogen. Approximately 0.5 g of frozen tissue powder was extracted twice with 3 ml of ice-cold 6% perchloric acid. After the centrifugations the combined supernatant was neutralized to pH 6.0 with 3.75 M K_2CO_3 containing 0.5 M triethanolaminehydrochloride. Neutralized perchloric acid extracts were used for enzymatic assays of lactate (Garavito and Berger 1970), pyruvate (Czok and Lamprecht 1970), α -glycerol-3-phosphate (Hohorst 1970), diacylglycerol phosphate (Bücher and Hohorst 1970), 3-hydroxybutyrate (Williamson and Mellby 1970), acetone (Meßanby and Williamson 1970), adenosine 5-triphosphate (Lamprecht and Trautbold 1970), adenosine 5-diphosphate (Jaworek *et al.* 1970), adenosine 5-monophosphate (Jaworek *et al.* 1970) and inorganic phosphate (Chen 1956).

$(\text{Na}^+ - \text{K}^+)\text{-stimulated ATP-ase}$ For the determination of $(\text{Na}^+ - \text{K}^+)\text{-stimulated ATP-ase}$ (EC 3.6.1.3) the rats were decapitated after 1 or 7 days at 40.8 kPa and their livers rapidly transferred to ice-cold 0.15 M sucrose containing 1 mM EDTA Tris buffer pH 7.4. The determination of ATP-ase proceeded exactly according to Bernstein *et al.* (1973). A 600 mg liver sample was homogenized with the same medium in glass homogenizer with a motor-driven Teflon pestle. Because activity determinations of total homogenate



3. Adenine nucleotide concentrations and phosphorylation state in the liver after various periods in hypoxia (40.8 kPa and 8 O₂). Experimental conditions and symbols as in Fig. 1. Values are means \pm SE from 4-6 separate experiments. ATP: adenosine-5-triphosphate; ADP: adenosine-5-diphosphate; P: adenosine-5-monophosphate; P_i: inorganic phosphate; ATP/ADP + P_i: phosphorylation state.

effect on the ratio though both the hydroxybutyrate and acetoacetate concentrations are increased especially after complete fasting (for 4 h (data not shown).

Adenine nucleotides. The results are presented in Fig. 3. In acute severe hypoxia the ATP concentration decreased ($p < 0.001$), and this decrease was still significant in chronically poxic animals after seven days ($p < 0.01$). No significant differences were observed between these acutely and chronically hypoxic animals ($p < 0.1$). A simultaneous increase ($p < 0.01$) in the inorganic phosphate concentration was observed in hypoxia. The ADP and AMP concentrations did not change significantly in any instance. The phosphorylation state (ATP/ADP + P_i) decreased both in acute ($p < 0.001$) and chronic hypoxia of seven days ($p < 0.02$), though there was also a significant difference between these two values ($p < 0.01$). The total amount of hepatic adenine nucleotides decreased ($p < 0.001$) in acute hypoxia, no differences being observable between these values and those recorded after hypoxic periods of one or seven days.

(Na⁺-K)-stimulated ATPase activity in the 48 000 g sediment did not change significantly in hypoxia after one day at 40.8 kPa. It was lower after 7 days in hypoxia when compared with the *ad libitum* control values, but the difference between the hypoxic and the pair-fed control activities was not significant (Table I).

The serum T concentration was 45.8 ± 4.0 nmol/l in the *ad libitum* control group, 49.3 ± 9.4 in the pair-fed control group and 35.6 ± 4.4 in the hypoxic group after seven days at 40.8 kPa. These values are means \pm S.D. from 7-8 different experiments, and the significance between the *ad libitum* control and hypoxic values is $p < 0.1$ and between the pair-fed and hypoxic values $p < 0.05$.

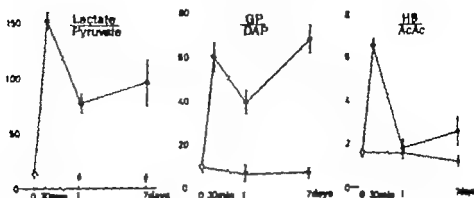
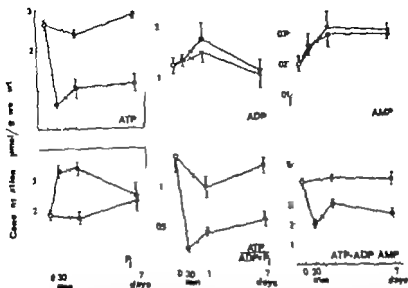


Fig. 1. Lactate/pyruvate, *sn*-glycerol-3-phosphate/dioxyacetone phosphate and 3-hydroxybutyrate/acetate ratios in the liver after various periods in severe hypoxia (40.8 kPa and 8% O₂). Experimental conditions and symbols as in Fig. 1. Values are means \pm S.E. from 4–5 separate experiments.

ratio was mainly due to change in the lactate concentration (see Fig. 1). The hepatic pyruvate concentration was 0.137 ± 0.003 (mean \pm S.D., $n=9$) $\mu\text{mol/g}$ wet weight in the controls and 0.098 ± 0.041 (mean \pm S.D., $n=6$) after 30 min of hypoxia (10% O₂). In severe hypoxia the lactate/pyruvate ratio increased by a factor of 11.2 ($p < 0.001$) after 30 min under 8% O₂ and 5.8 ($p < 0.001$) after one day and 7.0 ($p < 0.001$) after seven days at 40.8 kPa prior to the 30 min period in the respirator under 8% O₂ (Fig. 2). The hypoxic ratios were smaller at one day or seven days at 50.5 kPa or 40.8 kPa than in acute hypoxia with a corresponding partial pressure of oxygen. When the animals were removed from 40.8 kPa to 10% O₂ instead of 8% O₂, the results were about the same as at 50.5 kPa followed by 30 min with 10% O₂. Pair-feeding, in effect food restriction of the animals, resulted in no significant changes in the ratios (Fig. 1 and 2).

***sn*-Glycerol 3-phosphate/dioxyacetone phosphate ratio** The ratio increased by a factor of 5.6 ($p < 0.001$) in acute moderate hypoxia and by 2.8 ($p < 0.05$) after seven days in moderate hypoxia, this figure not being significantly different from the acute value ($p < 0.1$). The ratio was lower after the adaptation period of 24 h than in acute hypoxia, however ($p < 0.05$) (Fig. 1). The dioxyacetone phosphate concentration remained almost constant in spite of large changes in the *sn*-glycerol-3-phosphate/dioxyacetone phosphate ratio. The hepatic dioxyacetone phosphate concentration was 0.043 ± 0.007 (mean \pm S.D., $n=10$) $\mu\text{mol/g}$ wet weight in the control animals and 0.038 ± 0.010 (mean \pm S.D., $n=8$) in the animals kept in hypoxia (10% O₂) for 30 min. The transient change in the ratio noted during the first day in severe hypoxia did not prove significant ($p < 0.1$) (Fig. 2).

3-hydroxybutyrate/acetate ratio The ratio increased by a factor of 2.7 ($p < 0.05$) in acute hypoxia under 10% O₂ and 4.2 ($p < 0.001$) under 8% O₂. In acute hypoxia the increase in the 3-hydroxybutyrate/acetate ratio was due to an increase in the 3-hydroxybutyrate concentration. The hepatic acetoacetate concentration was 0.035 ± 0.004 (mean \pm S.E., $n=4$) $\mu\text{mol/g}$ wet weight after 30 min in hypoxia (10% O₂) and 0.039 ± 0.005 (mean \pm S.E., $n=4$) in the control animals. During chronic hypoxia the ratio decreased permanently near the control values, and no differences between the control and hypoxic ratios could be observed after either one day or seven days of adaptation (Fig. 1 and 2). Pair-feeding



1. Adenosine nucleotide concentrations and phosphorylation state in the liver after various periods in low hypoxia (40.8 kPa and 8% O_2). Experimental conditions and symbols as in Fig. 1. Values are means \pm SD from 4-6 separate experiments. ATP: adenosine-5-triphosphate; ADP: adenosine-5-diphosphate; AMP: adenosine-5-monophosphate; P: inorganic phosphate; ATP/ADP + P: phosphorylation state.

effect on the ratio, though both the hydroxybutyrate and acetoacetate concentrations are increased especially after complete fasting for 24 h (data not shown).

Adenosine nucleotides. The results are presented in Fig. 2. In acute severe hypoxia the ATP concentration decreased ($p < 0.001$), and this decrease was still significant in chronically hypoxic animals after seven days ($p < 0.01$). No significant differences were observed between these acutely and chronically hypoxic animals ($p < 0.1$). A simultaneous increase ($p < 0.01$) in the inorganic phosphate concentration was observed in hypoxia. The ADP and AMP concentrations did not change significantly in any instance. The phosphorylation state (ATP/ADP + P) decreased both in acute ($p < 0.001$) and chronic hypoxia of seven days ($p < 0.02$), though there was also a significant difference between these two values ($p < 0.01$). The total amount of hepatic adenosine nucleotides decreased ($p < 0.001$) in acute hypoxia, no differences being observable between these values and those recorded after hypoxic periods of one or seven days.

■ **(Na⁺-K⁺)-stimulated ATPase activity in the 48 000 g sediment** did not change significantly in hypoxia after one day at 40.8 kPa. It was lower after 7 days in hypoxia when compared with the pair-fed control values, but the difference between the hypoxic and the pair-fed control activities was not significant (Table 1).

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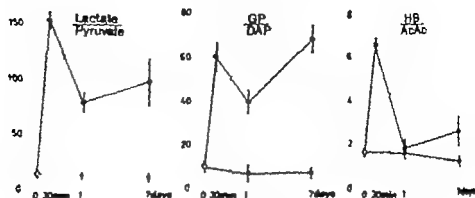


Fig. 2. Lactate/pyruvate *sn*-glycerol-3-phosphate/dioxyacetone phosphate and 3-hydroxybutyrate/acetate ratios in the liver after various periods in severe hypoxia (40.8 kPa and 8% O₂). Experimental conditions and symbols as in Fig. 1. Values are means \pm S.E. from 4-5 separate experiments.

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3-Hydroxybutyrate/acetate ratio. The ratio increased by a factor of 2.7 ($p < 0.05$) in acute hypoxia under 10% O₂, and 4.2 ($p < 0.001$) under 8% O₂. In acute hypoxia the increase in the 3-hydroxybutyrate/acetate ratio was due to an increase in the 3-hydroxybutyrate concentration. The hepatic acetate concentration was 0.035 ± 0.004 (mean \pm S.D., $n=4$) $\mu\text{mol/g}$ wet weight after 30 min in hypoxia (10% O₂) and 0.039 ± 0.005 (mean \pm S.D., $n=4$) in the control animals. During chronic hypoxia the ratio decreased permanently to near the control values, and no differences between the control and hypoxic ratios could be observed after either one day or seven days of adaptation (Fig. 1 and 2). Pair-feeding

observed during seven days in hypoxia by measuring the serum T concentration. This observation is in agreement with the increased phosphorylation state during the adaptation period, since a decreased phosphorylation state has been previously observed in the hepatic carylate system of thyroxine-treated rats by Häslinen *et al.* (1971).

The most sensitive indicators for the effects of hypoxia appear to be the decrease in ATP content and ATP/P ratio. Variations in ADP and AMP content cannot be interpreted with certainty because of the rapid decomposition of these nucleotides. The total adenine nucleotide concentration (ATP + ADP + AMP) decreased in hypoxia. The degradation of adenine nucleotides beyond AMP is dependent on the activity of 5-nucleotidase and/or AMP-deaminase, leading to adenosine or IMP and in both cases eventually to inosine. Since ATP inhibits 5-nucleotidase (Baer *et al.* 1966) and inorganic phosphate inhibits AMP-deaminase (Kikufuku and Colowick 1956, Ronca-Testoni *et al.* 1970) the route via adenosine would be favoured in hypoxia. Adenosine, one of the most potent vasodilators, is active in the ischemic myocardium (Rubio *et al.* 1973). The degradation products of IMP and adenosine lead to inosine, hypoxanthine, xanthine and allantoin, and it is by this mechanism that the raised concentration of inosine in anoxia can be explained (Olsson 1970).

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TABLE I (Na^+ K^+)-stimulated ATP-ase activity in control and hypoxic liver fractions (40 000 g and The hypoxic periods consisted of one or seven days at 40.8 kPa. Values are means \pm S.D. the number of experiments indicated in parentheses.

	Activity	
	nmol/min/g wet wt.	nmol/min/mg prot.
Control		
fed <i>ad lib</i>	315 \pm 38.0 (3)	38.2 \pm 4.4 (3)
fasted (one day)	343 \pm 126 (3)	37.2 \pm 22.0 (3)
Hypoxic (one day)	367 \pm 177 (3)	32.5 \pm 7.4 (3)
Control		
fed <i>ad lib</i>	494 \pm 144 (5)	41.6 \pm 10.9 (5)
pair-fed (seven days)	339 \pm 8.3 (3)	29.8 \pm 10.9 (3)
Hypoxic (seven days)	286 \pm 114 (8) ^a	30.5 \pm 6.7 (8) ^b

a = $p < 0.05$ as compared with the *ad lib.* control values of the same group.

b = $p < 0.1$ as compared with the *ad lib.* control values of the same group.

Discussion

The results demonstrate a significant adaptation of the energy metabolism to hypoxia: the cellular level in one day when evaluated from the redox state in the cytosol and mitochondria. The partial recovery of the initially high lactate/pyruvate ratios and lact concentrations (data not shown) during the adaptation period of seven days probably indicate diminished anaerobic glycolysis during the adaptation period. However no simultaneous decrease in ATP content occurred, and the phosphorylation state increased during the adaptation period.

The slight increase in the ATP concentration concomitantly with the decrease in lactate/pyruvate ratio during adaptation may be due to rapid mechanisms used to improve oxygen transport to the tissues or to reduce the consumption of ATP itself. Adaptive mechanisms which may be of significance in elevating the tissue oxygen concentration within a day include an increased hepatic artery blood flow (Fischer *et al.* 1960) and changes in oxygen-carrying capacity of the blood mediated by increased erythropoietin synthesis (Sjogren and Harris 1973), a rapid release of reticulocytes into the blood stream by erythropoiesis (Fruhman and Fischer 1962) and an increase in the red cell 2,3-diphosphoglycerate concentration (Lefant *et al.* 1968). (Na^+ K^+)-stimulated ATP-ase probably contributes significantly to the total ATP consumption of hepatic tissue, as has been demonstrated at least after prolonged ethanol administration (Bernstein *et al.* 1973) and thyroxine treatment (Lefant *et al.* 1973). A decrease in (Na^+ K^+)-stimulated ATP-ase activity in anoxic isolated perfused heart has been reported in one study (Balasubramanian *et al.* 1973). However the present results suggest that mechanisms other than the modulation of the activity of (Na^+ K^+)-stimulated ATP-ase are responsible for the adaptation of the cellular ATP consumption to hypoxia.

Inhibition of the uptake of ^{125}I by the thyroid gland, the concentration of thyroxine in the gland and the secretion of iodine by the gland within a few days in hypoxic hypoxia has been observed by Galton (1972). In the present case a slight inhibition of thyroid function

The effect of vigorous physical activity at work on serum lipids with a special reference to serum high-density lipoprotein cholesterol

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Abstract

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serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous were compared with those of electricians. The lumberjacks had significantly higher serum HDL-cholesterol and significantly lower triglyceride and free fatty acid concentrations but there were no significant differences in total cholesterol levels. The favourable effect of vigorous physical activity at work on lipid metabolism, however, epidemiologically not seen, obviously due to negative risk factors in lumberjacks, i.e.

low serum cholesterol, high density lipoproteins, serum physical activity, work activity

The distribution of plasma lipoprotein cholesterol may be influenced by the level of habitual physical activity in leisure time so that very active persons have higher high-density lipoprotein (HDL) levels (Carlson and Manfredi 1964, Lopez *et al.* 1974, Wood *et al.* 1976), but any reports about the effect of vigorous physical activity at work on the HDL-cholesterol have not been published. High concentration of HDL may be protective against coronary heart disease (CHD) to some degree (Nilkkilä 1953, Carlson and Eriksson 1975, Berg *et al.* 1976). Prospective studies have shown an increased incidence of ischemic heart disease with even mildly raised serum levels of cholesterol (Tromb *et al.* 1967) and triglycerides (Carlson and Böttiger 1972). We have compared the serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous with those of a group of ordinary electricians.

Material and methods

Subjects. All subjects completed questionnaire on their normal diet, on smoking habits and on alcohol intake before.

Lumberjacks (Group A, 12). Included in this group were only men who had worked at least two years as full-time lumberjacks and who were clinically healthy

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Key words: cholesterol, high density lipoproteins, serum, physical activity, work activity.

The distribution of plasma lipoprotein cholesterol may be influenced by the level of habitual physical activity in leisure time so that very active persons have higher high-density lipoprotein (HDL) levels (Carlson and Manfredi 1964, Lopez *et al.* 1974, Wood *et al.* 1976), and any reports about the effect of vigorous physical activity at work on the HDL-cholesterol have not been published. High concentration of HDL may be protective against coronary heart disease (CHD) to some degree (Nikkilä 1953, Carlson and Ericsson 1975, Berg *et al.* 1976). Prospective studies have shown an increased incidence of ischemic heart disease with even mildly raised serum levels of cholesterol (Tunstall *et al.* 1967) and triglycerides (Carlson and Bolliger 1972). We have compared the serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous with those of a group of ordinary electricians.

Material and methods

Subjects. All subjects completed questionnaire on their normal diets, on smoking habits and on alcohol intake.

Lumberjacks (Group A, 12). Included in this group were only men who had worked at least two years as full-time lumberjacks and who were clinically healthy.

TABLE I Age and physique of lumberjacks and electricians.

Group	Age (years)	Weight (kg)	Height (cm)	Weight Height - 100
A				
lumberjacks n = 12	42.4 ± 12.3 (23-57)	76.0 ± 15.8 (64-120)	172.7 ± 4.8 (163-181)	1.04 ± 0.17 (0.92-1.48)
B				
electricians n = 15	46.5 ± 7.6 (33-58)	83.0 ± 9.1 (69-101)	179.3 ± 6.5 (166-188)	1.05 ± 0.09 (0.95-1.21)

Electricians (Group B, n = 15). Their occupational physical activity was low or moderate and they without regular leisure time exercise.

The age, weight, height and relative weight of the lumberjacks and the electricians are shown in Table 7/12 lumberjacks and 5/15 electricians were smokers. All had normal diet and alcohol consumption low or moderate. The mean working time of the lumberjacks in this occupation was 13.9 years.

Chemical methods. Venous blood was drawn after fasting overnight 12-16 h before the test. All worked normally on the previous day. Plasma was separated by low speed centrifugation. Low density (LDL) and very low density lipoproteins (VLDL) were precipitated with a polyethylene-glycol solution (PEG-6000, final concentration 12%) and HDL was determined from the supernatant as described earlier (Vuokari 1976). Cholesterol was measured by a modification of the method of Badier and Bores (1967). Triglycerides were measured by the method of Carlson (1963). Free fatty acids were determined according to Mlakar Devic (1973).

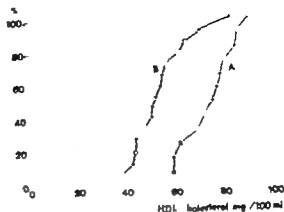
Results

Plasma lipid concentrations in each group are shown in Table II. Plasma total cholesterol concentrations were somewhat higher in the lumberjacks, but this difference was not statistically significant. This was obviously due to the higher HDL-cholesterol concentration ($p < 0.001$) since there was no difference in (VLDL + LDL)-cholesterol values. The cumulative distributions of HDL-cholesterol values are shown in Fig. 1. Three lumberjacks were overweight (relative weights 1.16, 1.25-1.48) had the lowest HDL-cholesterol concentrations (59.1, 59.6, 62.1 mg/100 ml). HDL-cholesterol levels of other lumberjacks were close

TABLE II. Mean plasma lipid and free fatty acid concentrations.

Group	Total cholesterol mmol/l	HDL cholesterol mmol/l	(VLDL + LDL)- cholesterol mmol/l	Triglycerides mmol/l	Free fatty acid mmol/l
A					
lumberjacks	5.79 ± 0.83	1.93 ± 0.28	3.86 ± 0.87	0.60 ± 0.4	0.39 ± 0
B					
electricians	5.20 ± 1.21	1.42 ± 0.31	3.78 ± 1.23	0.92 ± 0.41	0.51 ± 0
Statistical difference (t-test) between A and B	n.s.	$p < 0.001$	n.s.		

1. Cumulative distribution of HDL-cholesterol. Group A (lumberjacks), B (electrician), overweight ($\text{kg}/180 > 1$ HD); O, alcohol consumption moderate.



is than normal. There was no correlation between alcohol intake habits and HDL-cholesterol. There were two mildly hypercholesterolemic men in group A (serum cholesterol mol/l). Plasma triglyceride level of the lumberjacks was lower than that of the electricians. Free fatty acid concentrations were lower in lumberjacks as well.

Discussion

There are many studies concerning the effect of physical exercise in leisure time on serum lipids. The influence of physical exercise on serum triglyceride level has been consistently variable (Hollody *et al.* 1964, Haver *et al.* 1972, Wood *et al.* 1976). Plasma triglyceride concentration and age are positively correlated in sedentary subjects but not in athletes (Wier *et al.* 1972). A program of vigorous exercise has been followed by modest reduction in serum cholesterol concentration (Kilbom *et al.* 1962, Mann *et al.* 1969, Wood *et al.* 1976), while in other studies no such change was observed (Hollody *et al.* 1964). There are only a few reports concerning the effect of physical activity at work on serum lipids. No differences in serum cholesterol and triglyceride concentrations were found in different occupation groups (Carlson and Lindstedt 1968). The persons with high physical activity in leisure time have higher serum HDL-cholesterol concentrations than normal persons (Carlson and Lantfeldt 1964, Lopez *et al.* 1974, Wood *et al.* 1976). This finding is prominent only after a considerable amount of regular and vigorous exercise. The concentration of HDL-cholesterol in the runners was clearly higher than in a group with normal physical activity when the amount of kilometres run was more than 70 km per week (Lehtonen and Vlikeri, *in press*). Runners expended an average of about 5 000 kcal/week more energy than physically inactive controls. Our group of lumberjacks was small, but serum HDL-cholesterol concentrations in this group were significantly higher than in physically inactive men. The work of lumberjacks is known to require more energy than any other occupation. Recorded energy expenditure figures range from 4 500 to 8 000 kcal per day (Karvonen and Turpeinen 1954, Karvonen *et al.* 1961).

According to our results the effect of vigorous work activity on serum HDL-cholesterol is similar to that of exercise in leisure time. There were no major differences

in the alcohol intake habits of these two groups that would explain the difference. In group of lumberjacks three men were obese and their HDL-cholesterol levels were lowest in the group but triglyceride concentrations were normal. Total cholesterol concentrations in lumberjacks were slightly higher than in electricians. This was obviously due to higher HDL-cholesterol. Free fatty acid levels were slightly lower in lumberjacks. Those with high physical activity in leisure time had normal serum cholesterol levels but high free fatty acid concentrations (Lehtonen and Vilkar, in press).

Many reports have supported the idea that relatively low levels of plasma HDL-cholesterol are associated with an increased risk of coronary heart disease (Nikkilä 1953, Castelli 1975, Carlson and Ericsson 1975, Berg *et al.* 1976) and an increased incidence of ischemic heart disease with even mildly raised serum levels of cholesterol (Truett *et al.* 1967). High triglycerides (Carlson and Böttiger 1972) has been shown. There is, however, no clear relationship between VLDL-triglycerides and HDL-cholesterol as summarized by Miller and Miller (1975) so that in fact the decrease of serum triglycerides and the increase of serum HDL-cholesterol may reflect the same phenomenon.

The effect of increased physical work activity on serum HDL-cholesterol and triglycerides is advantageous as evidenced by the results of this study. If extreme physical work activity prevents the development of coronary heart disease, the evidence could be found among lumberjacks. However, the lumberjacks who were initially free of coronary heart disease did not manage better but rather worse as regards the development of coronary heart disease than men with a less heavy occupation on the 10-year experience (Punsar and Karvonen 1976). This might be because other risk factors may promote the development of coronary heart disease in lumberjacks. Smoking is a risk factor of major importance and there are more smokers in the lumberjacks than in control occupational groups (Karvonen 1961). The effect of lower socioeconomic status and working in difficult circumstances may be deleterious. On the other hand, longshoremen with heavy work activity had a lower death rate from coronary heart disease than their counterparts (Paffenberger and Hale 1971). In Rissanen's (1976) study the favourable effect of physical work activity was also observed. Men in occupations demanding regular physical activity had less extensive involvement of coronary arteries with atherosclerosis than men in sedentary occupations. As a conclusion it was found in this small material that vigorous activity at work has a beneficial effect on lipid metabolism.

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Blood flow, calcium deposition and heat loss in reindeer antlers

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Abstract

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Antler blood flow was studied in a 2 year old male reindeer during the last half of the antler growth period using an electromagnetic flow probe chronically implanted around the superficial temporal artery. Arteriovenous (a-v) differences of calcium were measured on antler blood. The blood flow increased from 80 ml/min when the antler was half-grown to 100-120 ml/min when fully developed. Subsequently a reduction was observed towards shedding. Positive a-v plasma calcium differences (on average 0.2 mM) were recorded during the period of active growth. Two bulls maintained positive a-v calcium differences during a 48 hour starvation period in spite of reduced arterial calcium concentrations. Exercise to near exhaustion caused a 2°C rise in the rectal temperature. Antler blood flow was decreased immediately after exercise but returned to pre-exercise values usually within 5-10 min. Since no overshoot in antler blood flow was recorded during the hyperthermia it is concluded that variations in blood perfusion of the antlers are of great importance in the defence against hyperthermia during and after exercise.

In Northern Norway reindeer antlers begin to grow in March and are usually fully developed by the beginning of July. During the growth period the antlers are covered by a highly vascularized, velveted skin. This skin remains vascular until August when it is shed, exposing the bony brown-grey antler cores. During the mating season and winter the antlers are important intraspecific symbols of social rank (Espmark 1964, Henshaw 1969). The males shed their antlers in mid winter, the females a few months later. This delay enables the females, privileged by the presence of antlers, to secure food which the males have to labor to expose from underneath the snow cover.

Apart from this social aspect of antler function it has also been suggested that the vascularized antler skin has a function in defense against hyperthermia during the summer season (Stonehouse 1968). Direct evidence supporting this suggestion seems to be lacking and the proposal of Stonehouse (1968) has been seriously questioned on behavioural and phylogenetic grounds by Henshaw (1969). Wilk and Krog (1971) and Wilk *et al.* (1972) have also questioned the role of antlers in heat dissipation after measurements of antler temperatures under different physiological situations. In the present study in order to obtain

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1 Position of flowprobe and leads on the superficial temporal artery in the animal used for blood flow measurements.

For direct evidence as to the role of antlers in thermoregulation we have measured blood flow through the antlers and antler temperatures before and after exercise hyperthermia. The bony antler has approximately the same mineral composition as ordinary skeletal bone, and the shedding of the antlers represents a considerable loss of minerals from the body. Inefficient nutrition is known to retard antler growth (Bubenik 1966). To study deposition of calcium in antler bone we have measured the extraction of calcium from food during maintenance feeding and in starved animals.

Material and methods

Animal. The study was carried out on two domesticated, 2-year old male reindeer (*Rangifer tarandus tarandus*) of very gentle disposition. The animals were kept in 4-4 m haddock pens on light regime about 12 days in advance of moult. On May 1st they were moved to an outdoor area. They were fed polished timothy hay and occasionally given supplements of birch branches, hay and grain.

Carotid loops. were established in both animals 1 month before measurements started. The animals were anesthetized with sodium pentobarbital and intubated. The carotid artery was exposed and surgically ligated in a permanent skin loop on the throat. The loops have worked satisfactorily for more than 1 year despite repeated punctures.

Antler blood flow. An electromagnetic Nycotron blood flow probe (I.D. = 3 mm) was fastened on the superficial temporal artery in one of the bulls. Its half grown antlers as shown in Fig. 1. The bull was anesthetized and local anesthesia infiltrated subcutaneously in the incision area. The artery to the right antler was exposed through a 10 cm incision and dissected free from connective tissue. Several side branches which supply the skin of the shaft were ligated and the flow probe positioned around the artery just above the neck base. The probe was secured to surrounding connective tissue and the lead tunneled under the skin and taken out through a small incision behind the antler (Fig. 1). Local anesthetic application at the 3rd and 4th temporary prevented infection and insect repellent prevented disturbance from flies. Despite these precautions local infection forced us to remove the probe on July 23.

For measurements of blood flow the probe was connected to Nycotron recorder. Zero flow was obtained by compressing the artery here this traverses the cheek bone. To control that all side branches had been tied off, i.e. that the recorded blood flow represented the total flow through one antler, we checked *in vivo* flow was also zero when compression was applied by hand around the base of the antler. *In vivo* observation of the flow signal could not be performed, therefore the calibration factor offered by the manufacturer was used to obtain actual flow values.

Flow was recorded at weekly intervals from April 20, to June 25.

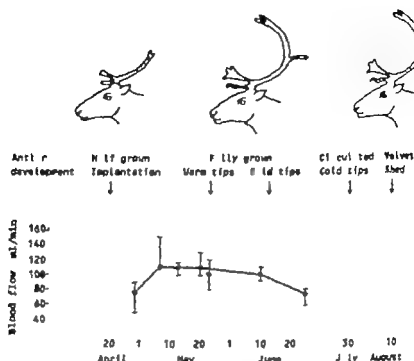


Fig. 2. Antler development and changes in antler blood flow (average and range of observation for a 20–60 min period of measurements). Blood flow after 48 hours of starvation.

Blood samples were taken at about one week intervals from both animals. Arterial blood was taken from the carotid loop. Venous blood was drawn from a catheter positioned in a vein at the base of antler. Due to the gentle disposition of our animals the samples were obtained with a minimum of motion and without the use of drugs. Plasma calcium was measured by atomic absorption spectrometry.

Body temperature (rectal) was measured at a 10 cm depth by an electronic thermometer. The temperature of the antler surface was measured by a thermistor covered with foam rubber during the last period of active growth (May 12) and near the time of velvet shedding (July 30).

Experimental

Antler development and blood flow were recorded at weekly intervals from one week after the flow probe had been positioned (April 20) until June 24. Care was taken to handle the animals as gently as possible to avoid both emotional stress and mechanical irritation of the growing antlers would decrease blood flow. Blood flow was measured repeatedly at each session. Arterial and venous blood was taken at the end of measurements.

The animals were starved, but received water for 48 hours beginning May 20. At the end of this period antler blood flow and arteriovenous blood samples were taken.

Temperature, flow measurements and heart rate were recorded before and after the animal had exercised to near physical exhaustion. The exercise was expertly conducted by a native Lapp reindeer handler using traditional harness and sledge.

Results

The antler from which blood flow was monitored developed to the same extent as the contralateral control antler. Fully developed,

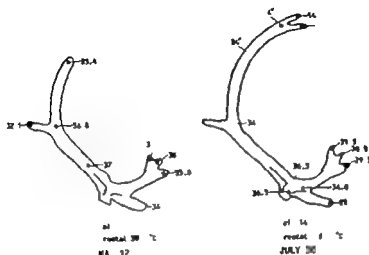


Fig. 3. Antler surface temperatures measured at rest in the period of active growth (May 12) and approximately 10 days before velvet shedding (July 30).

kg. Other males of the same age living under the same conditions developed antlers of similar size.

Antler blood flow and development are shown in Fig. 2. During each recording session we obtained a range of flow values. This probably does not reflect a truly normal situation as we frequently experienced that antler flow decreased if the animal was scared or if the antlers were touched. Although we tried to avoid this, we believe that the highest values are most representative for a normal antler flow. When the antlers were half grown the flow was 40-90 ml/min, when they were fully developed the flow was 110 to 160. This flow was maintained for the following two weeks while antler growth rate declined, and decreased rather slowly. Due to infection we were unable to measure blood flow in the antlers close to the

Table 1. Arterial plasma calcium and antler venous calcium differences (mM) in the fed state and after 48 h starvation. The antlers had reached their final size, but warm and soft tips indicated that active calcification was still going on. Animal No. 1: average and range of 3 observations from each antler. Animal No. 2: average and range (3 samples from one antler).

Animal no.	1	2
Arterial	2.70 (2.55-2.78)	2.76 (2.68-2.81)
Venous	0.29 (0.06-0.54)	0.14 (0.06-0.18)
Arterial	2.39 (2.24-2.57)	2.47 (2.41-2.54)
Venous	0.16 (+0.05-0.37)	0.20 (0.13-0.22)

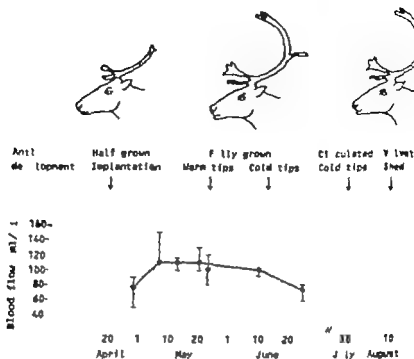


Fig. 2. Antler development and changes in antler blood flow (average and range of observations for a 20-60 min period of measurements). Blood flow after 48 hours of starvation.

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Body temperature (rectal) was measured at a 10 cm depth by an electronic thermometer. The temperature of the antler surface was measured by a thermistor covered with foam rubber during the last part of period of active growth (May 12) and near the time of velvet shedding (July 30).

Experimental

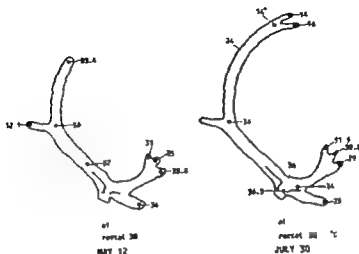
Antler development and blood flow were recorded at weekly intervals from one week after the flow probe had been positioned (April 20) until June 24. Care was taken to handle the animals as gently as possible so both emotional stress and mechanical irritation of the growing antlers would decrease blood flow. Blood flow was measured repeatedly at each session. Arterial and venous blood was taken at the end of 6 measurements.

The animals were starved, but received water for 48 hours beginning May 20. At the end of this period antler blood flow and arteriovenous blood samples were taken.

Temperature, flow measurements and heart rate were recorded before and after the animal had been exercised to near physical exhaustion. The exercise was expertly conducted by Otho Lapp (reindeer handler) using traditional harness and sledges.

Results

The antler from which blood flow was monitored developed normally and to the same extent as the contralateral control antler. Fully developed and dried each antler weighed



3 Antler surface temperatures measured at rest in the period of active growth (May 12) and approximately 10 days before velvet shedding (July 30).

k_g. Other males of the same age living under the same conditions developed antlers of other size.

Antler blood flow and development are shown in Fig. 2. During each recording session, we obtained a range of flow values. This probably does not reflect a truly normal situation, as we frequently experienced that antler flow decreased if the animal was scared or if the antlers were touched. Although we tried to avoid this, we believe that the highest values are the most representative for a normal antler flow. When the antlers were half grown the flow was 40-90 ml/min, when they were fully developed the flow was 110 to 160. This flow was obtained for the following two weeks while antler growth rate declined, and decreased thereafter. Due to infection we were unable to measure blood flow in the antlers close to the

As 1 Arterial plasma calcium and aortic systemic venous calcium differences (mM) in the fed state and after 48 h starvation. The aortas had reached their final size, but warm and soft tips indicated that active calcification was still going on. Animal No. 1: average and range of 3 observations from each aorta. Animal No. 2: average and range (3 animals from one aorta).

parameter no.	1	2
σ^2		
varied	2.70 (2.35-2.70)	2.76 (2.66-2.81)
ρ_{xy}	0.29	0.14
fixed	(0.06-0.54)	(0.08-0.18)
σ^2		
varied	2.29 (2.24-2.37)	2.47 (2.41-2.54)
ρ_{xy}	0.16	0.30
fixed	(-0.05-0.37)	(0.18-0.22)

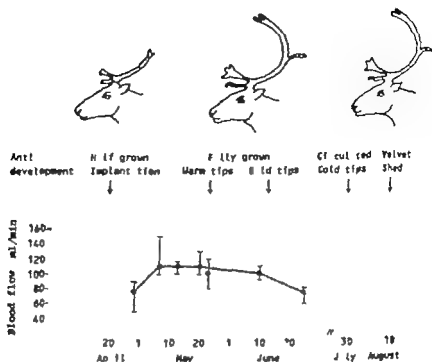


Fig. 2. Antler development and changes in antler blood flow (average and range of observations in a 20–60 min period of measurements). Blood flow after 48 hours of starvation.

Blood samples were taken at about one week intervals from both animals. Arterial blood was obtained from the carotid loop. Venous blood was drawn from a catheter positioned in a vein at the base of antler. Due to the gentle disposition of our animals the samples were obtained with a minimum of motion and without the use of drugs. Plasma calcium was measured by atomic absorption spectrophotometry.

Body temperature (rectal) was measured at a 10 cm depth by an electronic thermometer. The temperature of the antler surface was measured by a thermometer covered with foam rubber during the last part of period of active growth (May 12) and near the time of velvet shedding (July 30).

Experimental

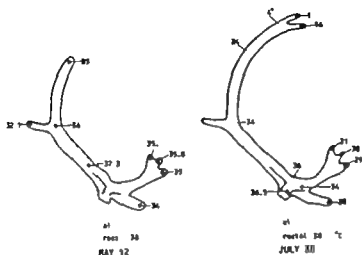
Antler development and blood flow were recorded at weekly intervals from one week after the flow probe had been positioned (April 20) until June 24. Care was taken to handle the animals as gently as possible, as both emotional stress and mechanical irritation of the growing antlers would decrease blood flow. Blood flow was measured repeatedly at each session. Arterial and venous blood was taken at the end of measurements.

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TABLE 1. Arterial plasma calcium and antler arterio-venous calcium differences (mM) in the fed state and after 48 h starvation. The antlers had reached their final size, but warm and soft tips indicated that active calcification was still going on. Animal No. 1: average and range of 3 observations from each antler. Animal No. 2: average and range of 2 antler samples from one antler.

Animal no.	1	2
arterial		
total	2.70 (2.53-2.78)	2.76 (2.68-2.81)
V	0.29 (0.06-0.34)	0.14 (0.08-0.18)
arterio-venous		
arterial		
venous	2.29 (2.34-2.37)	2.67 (2.41-2.96)
V	0.16 (+0.05-0.37)	0.28 (0.18-0.22)
arterio-venous		

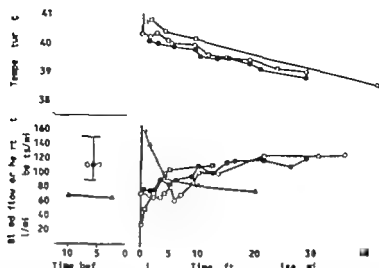


Fig. 4 Changes in rectal temperatures and blood flow during experiments with heavy exercise different occasions (\square \circ \bullet). Blood flow in pre-exercise periods: Range and approximate recorded flows during 10 min period. The heart rate ($-\Delta-$) was recorded manually by palpation carotid loops in one of the experiments.

time of shedding, but the low surface temperatures recorded on July 30, particularly in tips, clearly indicate that a further reduction in flow rate occurred towards shedding time (Fig. 3). The skin at the antler tips was circulated, but dry almost parchmentlike at this time.

The arterio-venous Ca differences varied considerably (Table I) with an average Δ of about 0.2 mM. Arterial plasma calcium levels were lowered after the 48 h of starvation but positive arterio-venous Ca differences were maintained in all pairs of samples over one (Table I).

The animal with flow measurement facilities was exercised on 3 occasions between 10 and 15 when the antlers were almost fully grown. Fig. 4 shows heart rate, body temperature and antler blood flow before and after exercise at ambient temperatures between -5 to $+5^\circ\text{C}$. A positive heat load is demonstrated by the profound post exercise hyperthermia (from normal 38.5 to about 40°C). The heart rate was 60–70 BPM at rest and about 100 immediately after exercise. The antler blood flow was from 30 to 70% of normal immediately after termination of exercise and gradually reached normal values during the following 1 hour. Values significantly above the pre-exercise values were only occasionally recorded. The surface temperatures of the antlers generally increased in proportion to the body temperature (about 2°C) when measured during the first 5 min after the exercise.

Discussion

Reindeer antlers begin as buds and grow into stalks which again divide by budding resulting in antlers with several distinct branches. Antler growth takes place primarily at the tips. The tips have a rich blood supply and show a high radiant temperature which can be felt by hand or visualized by thermovision (Wika and Krog 1971). The present results indicate that blood flow increased in proportion to the number of growth sites. A high flow rate

used for a considerable time after actual length-growth has ceased. This may be associated with thickness growth or antler ossification. With the approach of antler skin shedding, blood flow decreases, as is shown by direct measurements and indicated by measurements of surface temperatures 10 days before shedding.

Based on our measurements of blood flow and arteriovenous calcium gradients we can estimate the amount of calcium deposited during the approximately 100 days of growth. Flow of 100 ml/min and a gradient of about 0.20 mM give 90–100 g of calcium deposited, about 17% of the dry weight of cervid antlers is calcium (Bubenick 1966, Gebke 1973, Jernberg 1968). Our animal had an antler which weighed 900 grams indicating a total calcium deposit of 150 g. This compares favourably with our calculation and supports our contention that the highest rather than the average recorded blood flow is normal. After twelve the a-v differences might have been somewhat higher in the period prior to our measurements. The extraction of calcium by growing antlers is high enough to enable measurement, and cervid antlers might therefore be a valuable organ model for the study of calcium metabolism in rapidly developing bony tissue.

After a 48 h fast the calcium absorption from the digestive tract is probably reduced to a minimum and the plasma calcium concentration most likely maintained by bone resorption. 10–20 per cent reduction in arterial calcium levels at the end of the starvation period clearly indicated a limited calcium supply but the levels were still well within the normal range in reindeer (Björgholm *et al.* 1976, Hyvärinen *et al.* 1977). Irrespective of this decrease in plasma calcium concentrations positive arteriovenous differences across the antlers were maintained in both animals, indicating transport of calcium from skeleton to antlers during such conditions. Long term starvation or insufficient feeding is known to reduce antler growth (Bubenick 1966), but it is not known if Ca shortage is the main limiting factor under such conditions.

When reindeer are exercised to hyperthermia, heat dissipation is increased by panting and eventually eating of snow. If heat dissipation by the antlers is of functional significance vasodilatation and increased perfusion should be the result of positive heat loading. The present experiments demonstrated in the contrary that antler blood flow decreased as the animal approached thermal collapse, and remained reduced in the early phases of recovery. Heart rate and antler flow returned to preexercise levels within five to 20 min after the termination of work, while 30–40 min were needed to dissipate the heat load incurred during a work period. During the last 20–30 min of the hyperthermia period no overshoot in antler blood flow was recorded. Recently Wilson and Edrington (1978) reported evidence of the presence of adrenergic receptors in the antler arteries, and presence of well innervated vessels below the antler. The pronounced decrease in antler blood flow observed immediately after the end of the exercise can thus be explained as part of the increase in vasoconstrictor tone to skin during heavy exercise. Taylor (1966) found that the circulation through the carotid sinus may be used to regulate brain temperature by heat exchange between cool arterial blood in the cavernous sinus and arterial blood in the carotid rete. The carotid rete is present in most artiodactyls. The venous structures necessary for this heat exchange also seem to be present in reindeer since we found a well developed communication at the base of the antler between the superficial temporal vein and an orbital vein leading

towards the cavernous sinus. A similar venous arrangement has been reported in bighorn deer (Ohtakhi and Too 1974). Actively growing antlers have warm tips and the skin temperature, and therefore probably also venous temperature, on the branches and stems is a few degrees below body temperature even at ambient temperatures around 0°C (Fig. 1). It therefore seems doubtful whether cooling of arterial blood to the brain by antler venous blood can be of functional importance during the period of growth, when the growth ceases probably determines the flow. This might be different when the antlers are fully developed, however, since the surface temperatures then approach ambient temperature (Fig. 3) and cooled blood is returned to the head. In conclusion, the results of the present study demonstrate that antlers do not function as radiators for surplus heat during exercise hyperthermia. It seems doubtful whether antler venous blood can serve as a cooling medium for arterial blood in the carotid rete when antlers are in active growth, but it may do so when the antler is fully developed.

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Isometric and dynamic endurance as a function of age and skeletal muscle characteristics

By

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Abstract

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The aim of the study was to investigate the influence of age on endurance of human skeletal muscle. An attempt was made to correlate muscular performance at various ages with some morphological and enzymic characteristics of the muscle. Fifty healthy men, 22-65 years of age, with low daily physical activity (ark) volunteered for the study. Isometric and dynamic endurance were determined under standardized conditions and measured in relation to maximum strength, thereby correcting for individual as well as age differences in maximum strength. Biopsies taken from the quadriceps muscle were used for muscle fibre measurement, fibre area determinations, and measurements of some enzyme activities (Mg^{++} stimulated ATPase, myokinase (MK), lactate dehydrogenase (LDH), LDH isoenzymes). Maximum isometric and dynamic strength decreased in the older groups. No significant change was seen in isometric or dynamic endurance. Significant correlations are observed between endurance and fibre type distribution, fibre area, and LDH isoenzyme activities.

Key words: Fibre area, fibre type distribution, human skeletal muscle, isokinetic, lactate dehydrogenase.

Several studies have shown that maximum isometric strength of skeletal muscle decreases with age (Queletat 1836, Ulfand 1933, Asmussen and Heebell-Nielsen 1961). The effect of increasing age on muscular endurance is, on the other hand, less well documented. Petrofsky and Lind (1975 a, b) found that isometric endurance measured as performance time at 40 % of maximum isometric strength (MIS) increased with age and suggested that this was due to change in proportions between type I and type II fibres towards a higher proportion of type I fibres. This suggestion was made since type I (slow twitch) are generally held to be more fatigue-resistant than type II (fast twitch) fibres. Type II fibres are, on the other hand, associated with greater strength and higher speed of movements (for ref. see Burke and Edgerton 1975).

Hakkinen *et al.* (1975), studying the properties of the quadriceps muscle in a group of young men, have shown that a correlation exists between isometric endurance and percentage of type I fibres. Also dynamic endurance, measured as the ability to maintain tension output during repeated dynamic knee extensions, was found to correlate with the histochemical

properties of the quadriceps muscle (Thorstensson and Karlsson 1976). More recent studies have shown that lactate accumulation, total lactate dehydrogenase (LDH) as well as LD isozyme activities are of importance for dynamic endurance in the contracting quadriceps muscle (Tesch *et al.* 1978).

The results of the studies cited above thus suggest that the histochemical and biochemical properties of muscle are important factors for an individual's capacity to perform long lasting isometric or repeated dynamic contractions. The aim of the present study was to test this hypothesis by measuring muscular endurance in various age groups of a population of sedentary males, for which the relation between age and fibre type proportions and enzyme properties had already been determined (Larsson *et al.* 1978a). Since the quadriceps muscle was used for these histochemical and biochemical studies, this muscle was also used for studies of mechanical performance. The choice of the quadriceps muscle had an additional advantage because it has been shown that ageing processes, such as decline in muscle strength and muscle atrophy, occur relatively early in this muscle (Charles 1964; Serrano *et al.* 1968; Tomlinson *et al.* 1969).

Material and methods

50 healthy male subjects from 22 to 65 years of age volunteered for the study. The subjects were employees of an insurance company (clerks) and were in general minimally physically active, and all could be divided into groups I–II according to Saltin and Othman's (1968) classification of occupational and spare-time physical activity. On physical examination they were found to be without locomotor defects.

Height, weight, skinfold thickness, and skeletal breadth (femoral condylar and bityloid radii) were measured for each subject to estimate fat-free soft tissue weight (FFS) by the method of Hummer and von Döbeln (1971).

Maximum isometric strength (MIS) and isometric endurance, respectively, were determined for both legs simultaneously (Karlsson and Ölander 1972) by recording the force exerted when the subjects pressed their feet against a stiff bar equipped with a force transducer. MIS was taken as the highest force obtained during a series of five contractions. After three minutes rest, isometric endurance time was recorded, measured as the maximum time during which a tension level of 50% of MIS could be maintained.

Maximum dynamic strength and dynamic endurance were measured with a commercial isokinetic dynamometer (Cybex II, Lumex Inc., New York) in the left knee extensor muscles at a preset velocity of π rad/s (180 deg/s). The subject was sitting in an experimental chair with hip angle of π rad (90 degrees). The lower leg moved the lever of the dynamometer; the knee joint being aligned with the fulcrum of the lever. The subject was instructed to exert maximum force against the lever through its whole angular movement from $\pi/3$ rad to 0 rad (100 to 0 degrees, i.e. full knee extension). Maximum dynamic strength (MD) was defined as the highest value for peak torque from two knee extensions. Dynamic endurance was calculated from measuring 50 maximal contractions and determining the absolute (Nm) or relative (%) decline in peak torque from the mean of the three initial contractions to the mean of the three final contractions. Thus, dynamic endurance increased with decreasing absolute or relative decline. For detailed information of the apparatus and experimental set-up, see Thorstensson (1976), Thorstensson and Karlsson (1976), and Thorstensson *et al.* (1976).

The subjects in this paper are identical with those employed for two previous investigations of maximum strength, muscle histochemistry and enzyme activities in various ages (Larsson *et al.* 1978a, b) except for five men who were unable to participate here. Since the results of the previous study are discussed here as used for correlation with the mechanical performance data, a brief account of the methods used is given.

Two muscle biopsies were taken from the vastus lateralis muscle (Bergström 1962), one was used for histochemical analyses, the other for enzyme activity analyses. In all subjects, the muscle fibres were classified into type I (slow twitch) and type II (fast twitch) (Engel 1962). The average cross-sectional area of the two main fibre types as well as the relative cross-sectional area of each fibre type in the muscle were determined (Gollnick *et al.* 1972; Dubowitz and Brook 1973). In 40 of the subjects the type II fibres

TABLE I Height, body weight, and fat free soft tissue weight (FFS) of the different age groups.

Age group (yr)		Average age (yr)	Height (cm)	Body weight (kg)	FFS (kg)
0-29	10	26.1 ± 0.8	182.9 ± 2.0	73.8 ± 3.2	53.9 ± 2.4
30-39	10	35.3 ± 1.0	180.2 ± 2.1	78.1 ± 3.1	54.1 ± 2.6
40-49	8	42.6 ± 0.8	181.5 ± 1.3	77.6 ± 2.8	54.6 ± 2.0
50-59	12	54.5 ± 0.6	179.8 ± 1.3	79.2 ± 1.4	54.1 ± 1.4
60-65	10	61.6 ± 0.6	176.4 ± 2.5	75.2 ± 3.3	52.4 ± 2.4

Values are means ± S.E.

classified into A, B, and C subgroups (Brooks and Kaiser 1970, Debowitz and Brooks 1973). The activities of Mg^{++} associated ATPase, creatinase (CK), and lactate dehydrogenase (LDH) are determined fluorometrically (Loray and Passoureaux 1972). The LDH isoenzyme pattern was quantified by means of densitometric scanning (Ponary et al. 1974).

Stepwise multiple regression analyses have been performed. In data processing, the SPSS (Statistical package for the social sciences) (Nie et al. 1975) as used with MTS, MDS, isometric endurance and absolute or relative decline in dynamic strength as dependent variables. Independent variables were the anthropometric, histochemical, and biochemical variables together with subject's age. Pairwise deletion of missing data was used (Opocins 2, Nie et al. 1975). In each step in the analysis, the variable added to the regression equation is the one which makes the greatest reduction in the error sum of squares of the dependent variable and has the highest partial correlation with the dependent variable in proportion to the variables which have already been added to the equation. The last step (equation) given in Results is the step with minimum residual variance.

Antidromic means and standard errors were also calculated. The significance of linear correlation coefficients as tested from individual cases according to Snedecor and Cochran (1967).

Results

Anthropometric data in the different age groups are presented in Table I. A comparison of these groups with representative age-matched groups of the Swedish male population revealed a significant difference ($p < 0.05$) only in height in the 50-59-year-old groups, i.e. the subjects presented here were taller compared with 50-59-year-old representative Swedish males (179.8 cm and 174.3 cm, Levin, personal communication).

Summary of histochemical and biochemical results (Table II). The proportion of type II fibres decreased with age. Type II fibre subclassification revealed a decrease in percentage

TABLE II Fibre type distribution, fibre area ratios, and LDH isoenzyme activities in the muscle of the different age groups.

Age group (yr)		Average age (yr)	Fibre type distribution (% type I)	Fibre area ratio (type II/I area ratio)	LDH isoenzyme activities (arbitrary units g^{-1} min $^{-1}$)	
					M	H
20-29	10	26.1 ± 0.8	43.0 ± 3.1	1.30 ± 0.08	34 ± 5	10 ± 1
30-39	10	35.3 ± 1.0	36.8 ± 1.5	1.24 ± 0.09	44 ± 7	17 ± 4
40-49	8	42.6 ± 0.8	48.2 ± 5.1	1.08 ± 0.07	30 ± 5	17 ± 2
50-59	12	54.5 ± 0.6	51.7 ± 3.0	0.99 ± 0.05	20 ± 3	16 ± 3
60-65	10	61.6 ± 0.6	53.0 ± 4.5	0.99 ± 0.09	23 ± 3	14 ± 3

Values are means ± S.E.

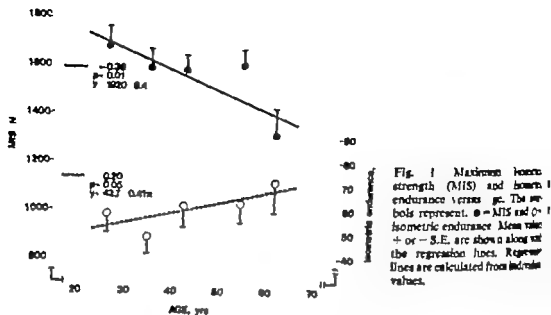


Fig. 1 Maximum isometric strength (MIS) and isometric endurance versus age. The symbols represent: \bullet = MIS and \circ = isometric endurance. Mean value \pm or $-$ S.E. are shown along with the regression lines. Regression lines are calculated from individual values.

of both type IIA and IIB fibres during ageing, while no change was seen in percentage of IIC fibres. Fibre area determinations, however, showed that the area of type I fibres remained unchanged while there was a decrease in type II fibre area. Consequently the II/I fibre area ratio and relative type II fibre area decreased from 1.30 to 0.99 and 61 \pm 44% in the 20-29 and 60-65-year-old group, respectively. The anaerobic M LDH activity decreased during ageing while no change was seen in the "aerobic" H LDH activity, resulting in a decreased M/H LDH activity ratio (Table II). The activities of Mg^{2+} stimulated ATPase and myokinase were, on the other hand, unchanged (Larsson *et al.* 1978 a).

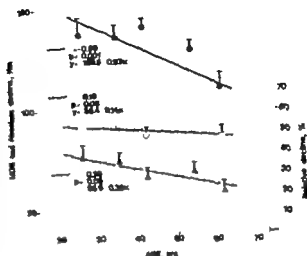
Maximum strength MIS (two-leg) and MDS (one-leg) followed a similar pattern during ageing, i.e. a marked decline was only seen in the oldest groups (Fig. 1 and 2). The decline in MIS and MDS, seen in old age, were observed to correlate significantly ($p < 0.001$) with the atrophy of type II fibres (Table III).

Endurance Isometric endurance, measured as the ability to maintain 50% of MIS, tended to increase with age (Fig. 1). Also dynamic endurance, i.e. the inverse of the absolute relative force decline during repeated isokinetic contractions, showed a slight increase with age (Fig. 2). However, the increase was not statistically significant in either case.

Simple correlations Some of the histochemical and biochemical muscle characteristics previously seen to change with age (Larsson *et al.* 1978 a), correlated significantly to isometric and dynamic endurance when all subjects irrespective of age were included. Isometric endurance, for instance, decreased with an increasing type II fibre area ($p < 0.05$). The relative force decline increased with an increasing proportion of type II and type IIA fibres as well as an increasing relative type II fibre area ($p < 0.05-0.01$). The absolute force decline increased with an increasing relative type II fibre area and increasing M LDH activity ($p < 0.05-0.01$).

Neither isometric endurance nor the relative force decline correlated with maximum strength, while the absolute force decline increased with an increasing MDS ($p < 0.05$).

Fig. 2. Maximum dynamic strength (MDS), relative decline of absolute decline versus age. The symbols represent: \circ MDS, \square relative decline and Δ absolute decline. Note that small errors decline corresponds to high dynamic endurance and vice versa. Mean values \pm S.E. are shown along with the regression lines. Regression lines are calculated from individual values.



Partial correlations: In simple as well as in multiple regression analyses, isometric endurance correlated significantly only with type II fibre area (Table III). The same general relationships seen in simple regression analyses for dynamic endurance were also seen in multiple regression analyses.

Discussion

Muscular endurance (isometric and dynamic) has been reported to decrease during ageing (Columbini *et al.* 1950, Burke *et al.* 1953, Simonson 1977). However in these studies, no correction was made for the decrease in maximum strength during ageing. The major observation in the present paper was that no decrease in muscular endurance could be seen with increasing age, when correcting for the decrease in maximum strength, if anything, endurance tended to increase.

So far few studies have demonstrated changes in isometric endurance with age. Petrofsky and Lind (1975 a, b) studied maximum handgrip strength and handgrip endurance (40% of maximum strength until the subjects could no longer maintain tension) in 100 industrial workers aged from 22 to 62 yrs. No significant changes were found in maximum strength or endurance although endurance tended to increase with age. However body weight increased with age and when multifactor analysis was used to delineate the independent contribution of weight to strength and endurance, maximum strength decreased and endurance increased with age. The present findings are thus in agreement with these data. Petrofsky and Lind suggested, on the basis of results of animal studies (Drabota and Gutmann 1963, Close 1964, Gutmann and Hamricková 1972), that the changes found in maximum strength and endurance with age might be due to changing proportions of type I and type II fibres giving a higher proportion of type I fibres with age. Since such a change in fibre proportions has been observed in human muscle (Larsson *et al.* 1978 a) the hypothesis appears tenable.

Although correlation ($p < 0.05$) between isometric endurance and type II fibre area ob-

TABLE III Step wise multiple regression analyses with independent variables presented in step 1 to 5. The simple regression coefficients with the dependent variable are also presented.

Dependent variable	Independent variables in stepwise order	Regression coefficients		Multiple correlation coefficient ²
		simple	partial ¹	
Maximum isometric strength (MIS)	1 Type II fibre area	0.17 **	0.18**	0.51**
	2. H LDH activity	788	764	0.53**
	3. M LDH activity	440	249	0.59**
Maximum dynamic strength (MDS)	1 Age	0.93	-0.90**	0.30**
	2. FFS	1.0	0.85	0.56**
	3. Type II fibre area	0.014 **	0.0065	0.59**
Isometric endurance	1 Type II fibre area	-0.01	-0.01	0.34
	2. Myokinase activity	-0.09	-0.10	0.38
Absolute force decline	1. M LDH activity	50.7*	42.0*	0.51**
	2. FFS	1.2*	1.1	0.60**
	3. Type I fibre area	-0.004	-0.006	0.63**
	4. Myokinase activity	-0.008	-0.10	0.67**
	5. % type II fibres	0.24	0.30	0.70**
Relative force decline	1. % type II fibres	0.21	0.29*	0.38*
	2. M LDH activity	14.6	13.1	0.45
	3. Myokinase activity	-0.009	-0.05	0.53
	4. Type I fibre area	-0.002	-0.003	0.57*
	5. FFS	0.27	0.28	0.60*

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

¹ Partial regression with the dependent variable when all independent variables are included in the regression equation.

² Multiple correlation with the dependent variable when all variables up to each step are included in the regression equation.

tained here is also in line with the results of Hultén *et al* (1975) who reported a negative correlation ($p < 0.01$) between isometric endurance and per cent type II fibres.

Until now no standardized measurements of dynamic muscular endurance and its relation to age have been published. In the present study a recently described test (Thorstensson and Karlsson 1976) was used to measure dynamic endurance in which the subjects performed repeated maximum knee extensions at a high angular velocity. The peak torque was registered and the relative (per cent) and absolute (Nm) force decline could be calculated and used as expression for dynamic endurance *i.e.* the dynamic endurance increased with decreasing relative or absolute force decline. The relative force decline in this material was found to be independent of MDS but increased with an increasing type II fibre population as earlier shown by Thorstensson and Karlsson (1976), although the correlation here was poorer.

It has been suggested that lactate accumulation (preferentially in the type II fibres) and postulated lactate translocation from type II to type I fibres are limiting factors for dynamic endurance measured by the method used here (Teach *et al* 1978). According to this hypothesis type I fibres would serve as a lactate recipient for lactate produced in the type II fibres. The increased dynamic endurance with an increasing relative area of the type I

res, found in the present study supports this hypothesis. The decreased endurance with increasing MLDH activity also indicates that the produced lactate is an important limiting factor in this type of exercise. The decline found in MLDH activity and in the proportion of type II fibres with age (Larsson *et al.* 1978 a) are in agreement with the tendency towards an increased dynamic endurance with age.

The same general relationships found between histochemical data and endurance data described by Hultén *et al.* (1975) (isometric endurance) and Thorstensson and Karlsson (1976) (dynamic endurance) have been found in the present study although the correlations are poorer. This was also true when multifactor analysis was applied to delineate differences age, muscle mass (FFS), maximum strength etc. The poorer correlations might, however, due to other ageing processes interacting with the muscle variables presented here, such as decreased intramuscular blood flow (Simonsen 1964), structural changes in the mitochondria (Lindler *et al.* 1978), and changes in the neuromuscular junction (Gutmann and Hanzliková 1972). The smaller variance in fibre type distribution observed here compared with those in the studies by Hultén and Thorstensson and their co-workers probably also contribute to the poorer correlations.

In summary no decline was seen in isometric or dynamic endurance during ageing (when measured in relation to maximum strength). Tendencies were, on the contrary observed towards increased endurance with age. These tendencies are in agreement with some of the changes seen in the histochemical and biochemical muscle characteristics during ageing, however maximum strength decreased during ageing, indicating a reduced isometric and dynamic endurance capacity when measured in absolute terms.

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Effect of metabolic inhibitors on phenylalanine influx in cerebral cortex slices from mature and immature rats

By

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Abstract

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Other investigations have shown that the transport of aromatic amino acids into cerebral cortex slices changes during ontogenetic development. In order to illuminate the possible influence of the developmental alterations of energy metabolism in the brain on the changes of the amino acid transport, the influx of phenylalanine into cerebral cortex slices of adult and 7-day-old rats was measured in the presence of metabolic inhibitors. Also the levels of lactate, ATP, ADP, AMP and creatine phosphate in the slices were measured. The used inhibitors cyanide, dinitrophenol and iodoacetate, which disturb main steps of energy metabolism, had very similar effects in both age groups on phenylalanine influx and on the levels of energy-rich phosphates. The correlation between the level of ATP and the influx of phenylalanine was positive in both age groups, but the slopes of the regression lines were different. If ATP supplies energy for amino acid transport, its utilization, rather than its availability is limiting factor in the slices from 7-day-old rats. Developmental changes in energy metabolism seem to have a significant role in the changes of amino acid transport during the maturation of the brain. Changes in the properties of cell membrane carriers and in the transmembrane ionic gradients may be more important.

Key words: cerebral cortex slices, transport of amino acids, metabolic inhibitors, brain development, phenylalanine.

The opinions of investigators differ as to how energy is derived for concentrative uptake of amino acids by cells. Energy-rich phosphates, ATP in particular, are thought to be involved the most often. Even if a direct coupling of the influx of amino acids with the hydrolysis of ATP has been disputed (Geck *et al.* 1974), there appears to be some kind of correlation between the rate of influx of amino acids and the intracellular level of ATP in slices of nervous tissue (Abelson and Scholefield 1962, Barbosa *et al.* 1968, Banny-Schwartz *et al.* 1971, Baker and Potashner 1973, Banny-Schwartz *et al.* 1974). This correlation is not, however, consistent under all incubation conditions. Energy-rich phosphates may also participate more indirectly in transport processes, e.g. by maintaining uneven distribution ratios for alkali metal ions between the intra- and extracellular fluids. The resulting electrochemical gradients may act as driving forces for transmembrane fluxes of amino acids (Riggs *et al.* 1958, Vidaver 1964, Eddy 1968). Kinetic analyses of our

earlier results on amino acid transport in brain slices have indicated *e.g.* that the *in vivo* velocities of the influx of aromatic amino acids into brain cortex slices and the half-saturation concentrations of the hypothetical carrier sites at cell membranes are smaller in slices prepared from 7-day-old rats than in slices from adult rats (Vahvelahti and 1972). The nature of energy metabolism in the brain changes appreciably during ontogenetic development (Berg 1971). The significance of anaerobic glycolysis (Rapp 1958, Takagaki 1974) and the hexose monophosphate shunt (Winick 1970) is greater in the immature brain. Piccoli *et al.* (1971) did not find in the slices from rat brain developmental changes of the levels of ATP which could explain the differences of amino acid transport in their study. Because ATP may have only an indirect role in the amino acid transport, it is possible that other developmental alterations in energy metabolism participate in the ontogenetic development of the transport processes.

Now we have continued our studies by comparing phenylalanine influxes into brain cortex slices of adult and 7-day-old rats incubated in the presence of iodoacetate, 2,4-dinitrophenol and cyanide, all of which are known to inhibit certain steps of cellular energy metabolism. The concentrations of energy rich phosphates and lactate in the incubation slices were also measured. The main purpose of the study was to find, if there are differences between the age groups 1) in the effects of the metabolic inhibitors on phenylalanine influx, or 2) in the correlation of phenylalanine influx to the levels of the metabolic metabolites.

Materials and methods

Chemicals

L-3-phenylalanine-2,3- ^3H (sp.act. 1.0 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. The metabolic inhibitors were sodium cyanide (NaCN) (J. T. Baker Chemicals N.V. Deventer), 2,4-(α)-dinitrophenol (DNF) (E. Merck AG, Darmstadt) and sodium iodoacetate (NaIA) (Fluka, Buchs SG).

Experimental procedure

Slices of brain cortex were prepared from adult male (180–200 g) and from 7-day-old (10–15 g) Sprague-Dawley rats at 4°C with a non-wetted tissue slicer of the Stadler-Riggs type. The thickness of the slices was 0.5 mm, and one side of each slice was 1 cm² brain surface. Two slices, weighing together about 100 mg, were used in each experiment. The slices were preincubated for 30 min at 37°C with shaking (80 oscillations/min, amplitude 2 cm) in a Warburg apparatus under continuous flow of pure oxygen in Erlenmeyer flasks containing 4.0 ml of Krebs-Ringer phosphate solution (pH 7.4, concentrations in mmol/L: NaCl, 118; KCl, 5.1; CaCl₂, 1.3; KH₂PO₄, 1.3; MgSO₄, 1.3; Na₂HPO₄/HCl-buffer, 10) (Umbreit *et al.* 1964) and glucose (10 mmol/l) and varying concentrations of the metabolic inhibitors. The control slices were preincubated without inhibitors.

After the preincubation, 1.0 ml of the incubation solution with [^3H] phenylalanine (1 nM concentration, 1.0 mCi/l) and unlabelled phenylalanine (final concentrations 0.02–2.0 mmol/l) was added to the flask and the incubation was continued for 5 min. The slices were then rinsed briefly on a filter paper in a funnel with 5 ml of cold Krebs-Ringer phosphate solution, weighed in 1.0 ml of cold 5% trichloroacetic acid (TCA) or in 1.2 ml of cold 0.6 N perchloric acid (PCA), and homogenized and centrifuged for 30 min at 10 000 g. The radioactivity of the TCA supernatants and that of the incubation solutions were determined with a Packard Tri-Carb scintillation spectrometer (Bray 1960) the counting efficiency having been determined with standard samples.

The concentrations of lactate, ATP, ADP and AMP were determined from the PCA supernatants enzymatically with Biochemica Test Combinations from Boehringer Mannheim GmbH (Hobart 1970, Jaworski *et al.* 1970) and the concentration of creatine phosphate by the method of Hobart and Rosenberg (1952).

1. Effect of metabolic inhibitors on the influx of phenylalanine into brain cortex slices of adult and 7-day-old rats, with varying concentrations of phenylalanine in the medium.

Age of rats	Conc. of phenylalanine in medium (mmol/l)	Control (nmol/kg)	NaCN 0.2 mmol/l (%)	NaCN 1.0 mmol/l (%)	DNP 0.1 mmol/l (%)	NaIA 1.0 mmol/l (%)
Adult	0.02	14.5 ± 0.6	132 ± 17	41 ± 10	61 ± 3	89 ± 14
	0.05	38.2 ± 2.0	103 ± 19	44 ± 6	59 ± 8	79 ± 7
	0.1	134.7 ± 25.5	115 ± 9	53 ± 19	55 ± 5	84 ± 5
	0.5	257.1 ± 18.1	104 ± 11	53 ± 14	68 ± 7	89 ± 10
	2.0	985.2 ± 161.3	71 ± 6	65 ± 18	67 ± 8	67 ± 6
7-day-old	0.02	24.3 ± 2.3	109 ± 7	57 ± 10*	52 ± 4	93 ± 4
	0.05	46.6 ± 3.2	121 ± 14	53 ± 14	58 ± 6	99 ± 14
	0.1	159.5 ± 20.2	109 ± 26	74 ± 27	56 ± 5	84 ± 8
	0.5	311.9 ± 43.4	107 ± 21	66 ± 14	62 ± 7*	84 ± 11
	2.0	1188.8 ± 121.2	82 ± 16	43 ± 6	67 ± 4	69 ± 14

Slices were preincubated for 30 min at 37°C under oxygen in Krebs-Ringer phosphate solution (7.4) with glucose (10 mmol/l) and with or without one of the metabolic inhibitors. [³H]phenylalanine in varying amounts of unlabelled phenylalanine were then added to give final concentrations of 0.02 and 0.02–2.0 mmol/l respectively and the incubations were continued for 5 min. The results mean of 4–6 experiments with their standard deviations. The control values are expressed as nmol/kg of sliced tissue and the other values as percentages of the controls. Abbreviations: NaCN—sodium cyanide, DNP—2,4-dinitrophenol, NaIA—sodium iodoacetate.

DNP, $p < 0.05$ when compared to corresponding controls in the two-tailed Student's *t*-test. In the age groups were compared, the control values differed significantly ($p < 0.01$) when the phenylalanine concentrations were 0.02 and 0.05 mmol/l in the medium.

Calculations

A amount of phenylalanine penetrated into the slices was calculated from the specific radioactivity of phenylalanine in the medium and the radioactivity of the TCA supernatant. Because of the short incubation period the incorporation of the label into proteins is negligible. According to thin-layer chromatograms developed with phenol water and *n*-butanol acetic acid, suc (5:1:4) less than 1% of it was metabolically degraded. In addition, the high medium-to-tissue ratio (30:1) ensured that the radioactivity of the incubation medium did not decrease significantly during the incubation (Vainanen and Oja 1972). The results are expressed as nmol/kg of wet weight of incubated tissue or as percentages of the initial concentrations. The statistical significance of the differences was calculated with the Student's *t*-test. The straight lines of regression of the phenylalanine influx on the ATP level were treated with the method of least squares. The slopes of the regression lines and the correlation coefficients with their 95% confidence limits and the analysis of the differences observed between them were carried out with common statistical methods (Sprengel 1961).

Results

Metabolic inhibitors and phenylalanine influx

During the 5-min incubation in all concentrations of phenylalanine the brain cortex slices of the 7-day-old rats took up slightly more phenylalanine from the medium than the slices from the adult rats (Table I). The influx was increasingly saturated at high phenylalanine concentrations. The initial influx into the slices from the adult rats appeared to be slightly enhanced by 0.2 mmol/l NaCN at low phenylalanine concentrations, but it was diminished by 30% at 2.0 mmol/l phenylalanine compared with the control (Table I). The influx was by about half by 1.0 mmol/l NaCN and 0.1 mmol/l DNP. The inhibitor caused

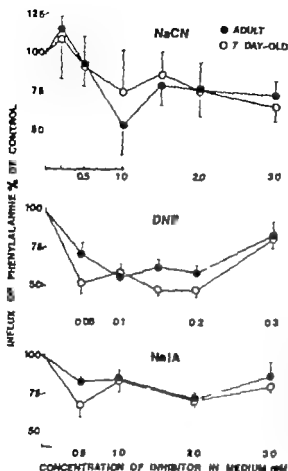


Fig. 1 Phenylalanine influx into brain slices of adult and 7-day-old rats in the presence of varying concentrations of metabolic inhibitors. The slices were incubated as indicated in Table I but the concentration of phenylalanine was constant (0.2 mmol/l) in the medium. Means of 4-6 experiments, and the standard deviations are expressed as the percentage of control wet weight of incubated tissue was used as reference.

by NaIA (1.0 mmol/l) was stronger at the highest than at the lowest phenylalanine concentration ($p < 0.05$). In the slices from the 7-day-old rats the effects of metabolic inhibitors were very similar to those in the slices from the adult rats.

When the phenylalanine concentration was kept constant (0.2 mmol/l) in the medium the concentrations of the inhibitors were varied, no striking differences between the groups were discernible (Fig. 1). The inhibition caused by NaCN reached its maximum at 1.0 mmol/l NaCN. DNP within the concentration range of 0.05-0.2 mmol/l inhibited influx by 30-45% and 40-55% in the slices from the adult and young rats, respectively. Influx was peculiarly greater in the presence of 0.3 mmol/l DNP than at lower concentrations. In the concentration range studied (0.5-3.0 mmol/l) NaIA inhibited influx by about 20-30%.

Metabolic inhibitors and the levels of the studied compounds

The concentration of lactate in the control slices was about four times greater in adult rats than in the 7-day-old ones (Table II). In the slices from the young rats the concentration significantly ($p < 0.01$) increased in the presence of NaCN or DNP, while in the slices from the adult rats NaIA decreased the level of lactate more in the slices from the adult rats than in those from the young ones.

(I. Effect of metabolic inhibitors on the concentrations of lactate, ATP, ADP, AMP and creatine phosphate in brain cortex slices of adult and 7-day-old rats.

	Metabolite	Control (nmol/kg)	NaCN 0.2 mmol/l ()	NaCN 1.0 mmol/l ()	DNP 0.1 mmol/l (*)	NaIA 1.0 mmol/l (*)
Adult	Lactate	8.86 ± 1.29	97 ± 6	91 ± 18	102 ± 8	16 ± 2
	ATP	1.25 ± 0.25	80 ± 10	48 ± 5	48 ± 6	59 ± 6
	ADP	0.86 ± 0.16	86 ± 16	68 ± 16	74 ± 15	65 ± 3
	AMP	0.46 ± 0.08	58 ± 11	57 ± 9	44 ± 11	34 ± 4
	CP	0.99 ± 0.12	20 ± 17	0*	37 ± 10*	59 ± 10*
7-day-old	Lactate	2.27 ± 0.78	140 ± 26	174 ± 34	235 ± 25	58 ± 43
	ATP	2.37 ± 0.46	91 ± 19	41 ± 7	37 ± 3	63 ± 2
	ADP	1.10 ± 0.17	99 ± 30	54 ± 5	53 ± 7	33 ± 7
	AMP	0.72 ± 0.04	60 ± 11	61 ± 14	42 ± 6	61 ± 10
	CP	1.20 ± 0.43	40 ± 5	4 ± 4	24 ± 7*	49 ± 14

Slices were incubated for 15 min at 37°C under oxygen in Krebs-Ringer phosphate solution (pH 7.4) glucose (10 mmol/l) and with or without one of the metabolic inhibitors. The results are means of 5-6 slices with their standard deviations. The control values are expressed as nmol/kg of wet weight of sliced tissue and the other values as percentages of the controls. Abbreviations: NaCN - sodium cyanide, DNP - 2,4-dinitrophenol, NaIA - sodium iodacetate, CP - creatine phosphate. * $p < 0.01$, $p < 0.05$ when compared to corresponding controls in the two-tailed Student's *t*-test. When control values of the two age groups are compared, the values of lactate, ATP and AMP differed scarcely ($p > 0.01$).

The concentrations of ATP and AMP in the control slices were higher in the young rats than in the adult rats, but the levels of ADP and creatine phosphate did not differ significantly (Table II). All the metabolic inhibitors diminished the levels of these compounds sharply in both the adult and young rats. The presence of 1.0 mmol/l NaCN, 0.1 mmol/l DNP or 1.0 mmol/l NaIA clearly diminished the levels of all the phosphate compounds. A concentration of 0.2 mmol/l NaCN was not very effective - only the decrements in ATP and creatine phosphate were significant.

Correlation between ATP level and phenylalanine influx

Linear correlation between the level of ATP and the influx of phenylalanine prevailed in 54 slices from both age groups (Fig. 2), the correlation coefficients differing from zero in 35 cases (Table III). The slopes of the regression lines of phenylalanine influx on ATP level were systematically greater in the slices from adult rats at the lower phenylalanine concentrations, many differences between the age groups being statistically significant ($p < 0.05$). The slopes were also dependent on the concentration of phenylalanine in the medium. Generally at higher medium concentrations a certain decrement in the ATP level coincided with a relatively smaller diminution of the phenylalanine influx. The slope of the regression line at the lowest phenylalanine concentration differed significantly ($p < 0.05$) from the slope at the highest concentration in both age groups.

There was no distinct correlation between the other phosphate compounds or lactate and the influx of phenylalanine.

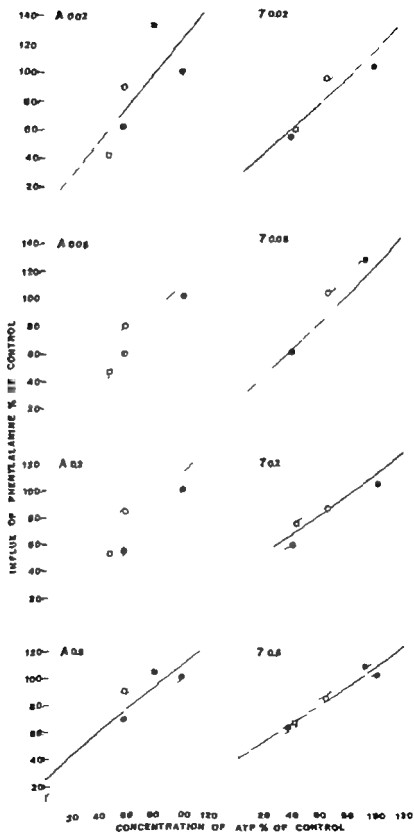
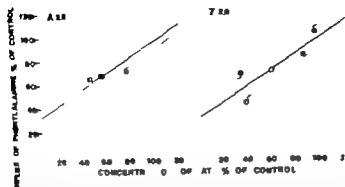


Fig. 2



2 Regression lines of phenylalanine influx and ATP level in brain cortex slices of adult and 7-day-old rats in the presence of metabolic inhibitors. The figures were drawn from the data in Table I and II. The vertical lines represent the standard error of the estimation of phenylalanine influx on ATP level. The age of animals (A = adult, 7 = 7-day-old) and the concentrations of phenylalanine (mmol/l) in the medium are used in the upper left-hand corner of each individual graph. Symbols: \bullet = control experiments, \blacksquare = 0.2 mmol/l α -cyano-L-proline, \square = 1.0 mmol/l sodium cyanide, \circ = 0.1 mmol/l dinitrophenol, \bigcirc = 1.0 mmol/l sodium acetate in the medium.

Discussion

relation of results

There are several alternative ways to express results when tissue slices are studied. We preferred to use the final wet weight of incubated slices as a reference because it could be more accurately assessed than any other possible reference. The share of [3 H] phenylalanine which penetrated only into the extracellular spaces of the slices has not been subtracted from the total influx since the size of the extracellular spaces cannot be easily measured with marker substances, in the presence of metabolic inhibitors in particular. We have earlier determined the swelling and the inulin space of rat brain slices for the same type of incubation conditions (Laakso and Oja 1976). The metabolic inhibitors increased the swelling on most occasions, and the slices from adult rats always swelled more. The inulin space was larger in the slices from 7-day-old rats under all conditions, but it proved to be a deceptively estimate for the extracellular space. When the present results were also arbitrarily calculated per initial fresh weight of the slices and the assumed inulin space was subtracted as a rough estimate for the extracellular space, the absolute magnitudes of the results were altered of course, but our inferences were not influenced by this trial.

When we calculated the share of saturable intracellular uptake in the total influx at different phenylalanine concentrations by using the transport constants estimated earlier by (K_t 0.66 and 0.22 mmol/l, V_{max} 101 and 44 mmol/kg/min for adult and 7-day-old rats, respectively) (Valveläinen and Oja 1977), it appeared to be about four-fifths at the low phenylalanine concentration of 0.02 mmol/l and only one-third at 2.0 mmol/l. At the lowest amino acid concentrations the effects of metabolic inhibitors mainly reflect changes in saturable intracellular transport, and at the highest concentrations in predominantly in the nonsaturable penetration into the slices.

TABLE III Correlation coefficients between the concentrations of ATP and phenylalanine and their (regression coefficients) of the least square regression lines of phenylalanine influx vs. concentration.

Age of animal	Conc. of phenylalanine in medium (mmol/l)	Correlation coefficient	Regression coefficient
Adult	0.02	0.72 (0.46-0.87)	1.22 ± 0.50
	0.05	0.87 (0.73-0.94)	1.05 ± 0.25
	0.2	0.79 (0.58-0.90)	1.04 ± 0.34
	0.5	0.82 (0.63-0.93)	0.86 ± 0.26
	2.0	0.90 (0.79-0.96)	0.64 ± 0.13
7-day-old	0.02	0.93 (0.84-0.97)	0.84 ± 0.13
	0.05	0.88 (0.74-0.95)	0.91 ± 0.21
	0.2	0.94 (0.86-0.97)	0.67 ± 0.11
	0.5	0.97 (0.93-0.99)	0.68 ± 0.07
	2.0	0.87 (0.72-0.94)	0.64 ± 0.16

The coefficients with their 95% confidence limits were calculated from the data presented in Table I as described in "Material and Methods". The regression lines are presented in Fig. 4.

Different from the corresponding value of the other age group ($p < 0.05$).

Effects of metabolic inhibitors

Cyanide restrains cell respiration by inhibiting cytochrome oxidase and therefore provokes a shift from aerobic to anaerobic metabolism (Keilin 1928; Stotz *et al.* 1938). It drastically affects the structures of nerve cells (Ibata *et al.* 1971) and may thus alter permeability of nerve cell membranes. A slightly enhanced influx of phenylalanine at 0.02 mmol/l cyanide in the presence of diminished levels of creatine phosphate and ATP indicate such permeability changes. Inhibition of the influx at 2.0 mmol/l phenylalanine probably results from diminution of the extracellular space (Laakso and Oja 1976). High NaCN concentration (1.0 mmol/l) reduced the influx at all phenylalanine concentrations and decreased both the intracellular transport and the extracellular space.

The main metabolic effect of DNP is the uncoupling of oxidative phosphorylation (Loomis and Lipmann 1948), but it may also stimulate a latent ATPase activity in mitochondria (Lardy and Wellman 1953). DNP also changes the conformation of membrane proteins of mitochondria and alters the permeability of mitochondrial membranes (Carafoli and Rossi 1967; Weinbach and Garbus 1968). It is not known what such conformational changes occur in neural plasma membranes. An unexpected increase in the phenylalanine influx at a DNP concentration of 0.3 mmol/l (see Fig. 1) could reflect an increased penetration of phenylalanine down to its concentration gradient in damaged cell membranes. This explanation is in accordance with the diminished inhibition provoked by DNP as the phenylalanine concentration increased (Table I).

Iodoacetate is a classical inhibitor of glycolysis at the level of glycerose 3-phosphate (Cori *et al.* 1948). The production of lactate is inhibited *in vitro* already at low concentrations (0.01 mmol/l) of iodoacetate (McMurray *et al.* 1957), whereas higher concentrations exert other diverse effects on cell metabolism. The uptake of the

ly, proteins, DNA and RNA may all be inhibited by iodoacetate (Heald 1953, Glazer Weber 1971 a). According to Guroff *et al.* (1961) iodoacetate does not alter the cell membrane permeability for passive diffusion, neither are the swelling or the size of the in space of brain cortex slices altered by iodoacetate (Laakso and Oja 1976). However in our present study the NaIA-induced inhibition of phenylalanine influx was significantly greater at the higher than at the lower phenylalanine concentration in both age groups. metabolic effects of iodoacetate may be augmented by high concentrations of phenylalanine because phenylalanine also inhibits glycolysis in brain slices (Woodman and Dawson 1961, Glazer and Weber 1971 b). Phenylalanine influx was less affected by 1.0 mmol/l NaIA than by 1.0 mmol/l NaCN or 0.1 mmol/l DNP. Also Banay-Schwartz *et al.* (1971) have reported that the NaIA-induced decrease in the level of ATP in brain slices is generally more pronounced than the concomitant decrease in the influx of amino acids.

relation between ATP level and phenylalanine influx

A correlation between the ATP level and the phenylalanine influx was more linear than expected since the inhibitors also produce other metabolic effects in addition to affecting ATP concentration. The positive correlation prevails also at high phenylalanine concentrations and this phenomenon suggests that ATP may play a role in the factors which influence the passive fluxes of amino acids in brain slices and the tissue swelling during incubation. Because the swelling of the brain slices during incubation parallels the increase of intracellular sodium (Piccoli *et al.* 1971), the role of ATP in the distribution of all metal ions in the slices may manifest itself in this finding. The intracellular swelling may increase when the level of ATP decreases, and the enlargement of intracellular volume may displace some extracellular fluid. So the amount of the amino acid diffused to the extracellular space diminishes and correlates to the ATP content of the slices.

From the above discussion one can conclude that the positive correlation between ATP level and phenylalanine influx also at low amino acid concentrations may be a secondary phenomenon and do not prove that ATP were a direct source of energy for the active transport of amino acids.

The regression lines of phenylalanine influx on the ATP level of the slices were systematically steeper for the adult than for the young rats at the low phenylalanine concentrations. The following explanations are possible: (1) The extracellular space in the slices from young rats is larger than that in the slices from adult rats (Laakso and Oja 1976), and the diffusion of phenylalanine into extracellular spaces should be independent of the ATP level. This difference in extracellular spaces between the age groups may not be large enough to constitute the only explanation however (2) Passive energy-independent diffusion across cell membranes could be faster in the slices from 7-day-old rats. We have determined earlier however that the transmembrane diffusion constants for phenylalanine are equal in slices from 7-day-old and adult rats (Avelaainen and Oja 1972). (3) In the control incubation the content of ATP was higher in the slices obtained from 7-day-old rats. After a similar percentage reduction in ATP there is still more ATP left in the slices of 7-day-old rats. This extra ATP is not, however able to support adequately the phenylalanine

influx. The breakdown rate of ATP in the brain tissue of young rats is slower than brain tissue of adult rats (Takagaki 1974). If ATP really in some way furnishes energy for amino acid transport, the rate of utilization of ATP in the slices of immature brain is a limiting factor for phenylalanine influx, but not the level of ATP as such.

Conclusions

The developmental changes in the influx of amino acids into cerebral cortex slices do not seem to be connected to the developmental changes in energy metabolism. Metabolic inhibitors which disturbed different steps in energy formation had very different effects on phenylalanine influx in the two age groups. The observed positive correlation between the ATP level in the slices and the influx of phenylalanine did not prove that ATP was a source of energy for amino acid transport. In any case, the level of ATP did not limit the influx of phenylalanine into the slices from immature brain. More probable reasons for the changes of amino acid transport in brain slices during development may be changes in alkali metal concentrations and distributions in the slices (Piccoli *et al.* 1972, Franck and Schoffeniels 1972), because as in other tissue preparations, the transport of amino acids in brain slices is at least partly dependent on optimal alkali metal concentrations (Margolis and Lajtha 1968). Furthermore, the changes in the properties of carrier systems of cell membranes are possible during the maturation of brain tissue.

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Hepatic triacylglycerol and fatty acid biosynthesis during hypoxia *in vivo*

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Abstract

KINNULA, V. L., M. J. SAVOLAINEN and I. HASSINEN. Hepatic triacylglycerol and fatty acid biosynthesis during hypoxia *in vivo*. Acta physiol scand. 1978 104: 148-155

Hepatic fatty acid biosynthesis and the activity of phosphatidate phosphohydrolase, the rate-limiting enzyme of triacylglycerol biosynthesis, were studied after hypoxic periods of 1 and 7 days under basal conditions at 40.8 kPa. Phosphatidate phosphohydrolase activity increased 2-fold in the soluble fraction of the liver after one day at 40.8 kPa but had returned to normal by 7 days. This was accompanied by a significant increase in hepatic triacylglycerol and *sn*-glycerol-3-phosphate. The phosphatidate phosphohydrolase activity increased continuously over 7 days in the pair-fed controls, probably due to the reaction on food. Measured as *in vivo* incorporation of $^3\text{H}_2\text{O}$ into lipids, the hepatic fatty acid synthesis increased somewhat in acute hypoxia, but returned to normal values during 7 days of hypoxia. Free fatty acids increased markedly after 24 h in the fasting controls (90%) with a smaller increase in the hypoxic group (30%) due to peripheral lipolysis. Hepatic glycogen stores decreased in the hypoxic fasting animals both after 1 and 7 days. It is concluded that hypoxia induces the accumulation of lipids in the liver at least partly as a consequence of an increase in phosphatidate phosphohydrolase activity in the soluble fraction of the liver.

Key words: Liver hypoxia, triacylglycerols, phosphatidate phosphohydrolase, fatty acid synthesis

In a previous paper (Kinnula and Hassinen 1978), we observed that severe hypoxia (40.8 kPa) causes triacylglycerol accumulation in the liver of the rat. Similarly ischemia or anoxia can lead to a lipid accumulation in the heart and aorta (Bryant *et al.* 1958, Scheraga and Brachfeld 1966, Briggs and Glenn 1976), and hypoxia-induced fatty liver in suckling rats has been observed (Chiodi and Bass 1969). One reason for this hypoxia-induced steatosis seems to be a reduction in fatty acid oxidation in the liver (Kinnula and Hassinen 1978), while other possible reasons might be lipolysis from the adipose tissue or increased lipogenesis in the liver. The rate of lipogenesis *in vivo* can be reliably studied by measuring the incorporation of tritium from $^3\text{H}_2\text{O}$ into hepatic lipids (Lowerstein *et al.* 1975). Our results also indicate that under certain conditions hepatic triacylglycerol accumulation is preceded by an increase in the activity of phosphatidate phosphohydrolase (E.C. 3.1.1.4) and it seems that this enzyme constitutes the rate limiting step.

erol and triacylglycerol by liver cells (Lamb and Fallon 1974 a, b, Savolainen 1977). Therefore further studies concerning hepatic triacylglycerol accumulation in hypoxia were conducted by determining the rate of fatty acid synthesis *in vivo* and the activity of phosphatidic phosphohydrolase in hypoxic rat liver.

Material and methods

Reagents. Standard reagents were obtained from E. Merck AG, Darmstadt, Germany. Coenzyme, glycerol, phosphatidyl dehydrogenase and lactate dehydrogenase are purchased from Boehringer GmbH, Mannheim, Germany and $^3\text{H}_2\text{O}$ from NEN Chemicals GmbH, Dreieichenhagen, Germany. Phosphatidic acid obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K.

Animals and hypoxic experiments. Adult Sprague-Dawley rats from the Department own colony are used. Both sexes were used, and the age of the animals varied between 3-4 months. Their food consisted of standard rodent chow (Astra-Ewos AB, Södertälje, Sweden). 3 groups of the animals were studied in each, forming an ad libitum-fed control, pair-fed control and hypoxic groups. For the lactate and α -glycerol-3-phosphate determinations the rats are anesthetized with pentobarbital (60 mg/kg, intraperitoneally). After cannulation of the trachea the respiration is controlled with a rodent respiration pump (David Apparets Company Model 640). The respiratory rate was fixed at 60 cycles per min, and the O_2 tension calculated from the equation $\log P_{\text{O}_2} = -1.51 + 0.735 \log \dot{V}_{\text{O}_2}$, where \dot{V}_{O_2} is the body weight of the animal in grams and P_{O_2} the total volume in millilitres (Kleemann and Radford (1971)). The animals breathed $100\% \text{ O}_2$ for 30 min in the control experiments and $8\% \text{ O}_2$ in nitrogen in the hypoxic experiments. In order to obtain stable O_2 concentration figures from partially adapted animals, the animals in one group of experiments were kept in a hypobaric pressure chamber at 40.8 kPa for 1 or 7 days before the 30-min period in the respirator under $8\% \text{ O}_2$. α -Glycerol-3-phosphate and lactate concentrations in the liver slices were measured after the 30 min period in the respirator. In studying fatty acid biosynthesis or phosphatidic phosphohydrolase activity hypoxia was induced in the hypobaric pressure chamber.

Phosphatidic phosphohydrolase. After a period of one or seven days in the hypobaric pressure chamber at 40.8 kPa the rats are decapitated and their livers removed immediately and placed in ice-cold 0.225 M Tris containing 1 mM EDTA and 30 mM Tris-HCl, pH 7.5. The liver was homogenized in 4 volumes of the same medium with glass homogenizers fitted with teflon pestle. The homogenate is centrifuged at 15 000 g for 20 min, after which the supernatant is centrifuged at 100 000 g for 60 min. After $(\text{NH}_4)_2\text{SO}_4$ sediment (0.23 g/ml, 0°C) and further centrifugation at 10 000 g for 20 min, the surface of the pellet was gently mixed with 1 ml of medium containing 0.25 M sucrose, 0.5 mM dithiothreitol and 20 mM Tris-HCl, pH 7.4 and then dissolved in this buffer. The phosphatidic phosphohydrolase activity of the $(\text{NH}_4)_2\text{SO}_4$ fraction is determined by the method of Honkala *et al.* (1973) as modified by Savolainen (1977). The release of inorganic phosphate released was determined by the method of Ames and Dubin (1960). The enzyme activity was expressed as moles inorganic phosphate liberated per min and g of wet weight.

Fatty acid synthesis *in vivo*. The rate of hepatic lipogenesis *in vivo* is measured by determining the incorporation of carbon from $^3\text{H}_2\text{O}$ into long-chain fatty acids as described by Lowenstein *et al.* (1975). In one series of experiments the animals are kept in the hypobaric pressure chamber at 40.8 kPa for seven days or pair-fed like the other period. At 9.00-9.30 a.m. on the eighth day they were anesthetized with ether and injected intracardially with 5 μCi of $^3\text{H}_2\text{O}$ in 0.9% NaCl. They are then returned to the hypobaric pressure chamber still at 40.8 kPa, and liver and blood samples are obtained exactly 60 min after injection under ether anaesthesia. In another series of experiments two groups of normal animals are injected with $^3\text{H}_2\text{O}$ one group is kept at normal atmospheric pressure, and the other group exposed to a pressure of 40.8 kPa. About 0.5 g of liver is weighed rapidly and homogenized in 19 volumes chloroform-methanol (2:1 by volume) using an Ultra-Turrax homogenizer. Fatty acids and non-saponifiable lipids are extracted and counted for radioactivity in a toluene-based scintillation solution (Lowenstein *et al.* 1975). The specific radioactivity of the body water of each animal was determined by measuring the tritium radioactivity of a sample of blood, and performing the calculations described by Amdur *et al.* and Sparks (1966).

Metabolic concentrations. At the end of the 30-min period of controlled ventilation in the respirator under approximately the same partial pressure of oxygen as in the hypobaric pressure chamber, one lobe of the liver is freeze-clamped by the technique of Wolfenbarger *et al.* (1966). After extraction of the lobe with 6 perchloric acid the supernatant is neutralized to pH 6.0 with 3.75 M K_2CO_3 con-

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1 Effect of hypoxia (40 kPa) on hepatic lipogenesis *in vivo*. For measurement of the rate of lipogenesis the rats were injected intracardially with 1.5 μ Ci (acute expts.) or 5 μ Ci (chronic expts.) of $^3\text{H}_2\text{O}$ and killed 60 min later during which time the animals referred to as hypoxic are kept at 40 kPa. Animals which had been at 40 kPa for 7 days or pair-fed or *ad libitum* control animals at normal atmospheric pressure were referred to as chronic expts. The radioactivity was determined from isolated lipid fractions (Lowenstein *et al.* 1975). Values are means \pm S.E. from 4 separate animals.

	Acute experiments		Chronic experiments		
	Control	Hypoxia	<i>Ad lib.</i> control	Pair-fed control	Hypoxic
Fatty acids (nmol of $^3\text{H}_2\text{O}$ incorporated/nec/g wet wt. of liver)	47.4 \pm 14.7	76.2 \pm 31.7	55.9 \pm 28.9	38.4 \pm 16.1	43.7 \pm 5.5
Triglycerides (nmol of $^3\text{H}_2\text{O}$ incorporated/nec/g wet wt. of liver)	30.0 \pm 11.0	25.3 \pm 9.1	27.8 \pm 7.9	37.7 \pm 20.7	35.5 \pm 16.7

acids, but the triacylglycerol concentration had not increased any further after 48 h (μ mol/g wet wt. of liver). The difference in triacylglycerol concentration between the pair-fed and hypoxic animals was significant ($p < 0.01$) after 7 days, but not after one day. *Fatty acid synthesis*. Acute hypoxia increased the hepatic lipogenesis, but this was not statistically significant (Table I). Seven day's hypoxia had no effects on the rate of fatty acid synthesis when compared with the normal or pair-fed controls (Table I).

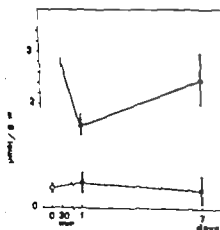


Fig. 2

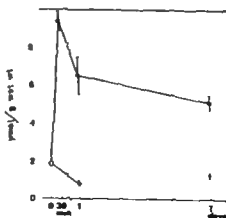


Fig. 3

Fig. 2. Effect of hypoxia on hepatic ac-glycerol-3-phosphate concentrations. For the 30-min period the animals were in a respirator under 8% O_2 and for the 1-day and 7-day expts. they were exposed to pressure of 40 kPa for the given length of time prior to the 30-min period in the respirator under 8% O_2 . Values are means \pm S.E. from 4-5 separate expts. Symbols as in Fig. 1.

Fig. 3. Effect of hypoxia on hepatic lactate concentrations. Values are means \pm S.E. from 4-5 separate expts. Experimental conditions and symbols as in Fig. 1 and 2.

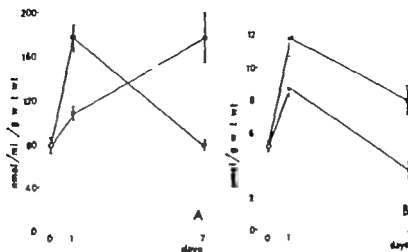


Fig. 1 Effect of hypoxia (40.8 kPa) on hepatic phosphatidate phosphohydrolase activity (A) and liver triacylglycerol concentration (B). Values are means \pm S.E. from 7–8 separate expts. Symbols: ○ control; ● hypoxic and ■—■ pair fed.

taining 0.5 M triethanolaminehydrochloride. Lactate and *sn*-glycerol-3-phosphate concentrations determined in the neutralized perchloric acid extract as described by Gawehn and Bergmeyer (1974). Hohorst (1970). In determining the hypoxic values of the following metabolites only the hypobaric chamber was used. Liver glycogen was determined from the freeze-clamped tissue according to van B (1965) and triacylglycerols according to Carlson (1963). Plasma free fatty acids were measured by method of Duncombe (1963) and blood glucose by that of Bergmeyer *et al.* (1970).

Statistical methods. The two tailed student's *t*-test for independent means was used for the statistical analysis, *P* values of <0.05 being considered to indicate statistical significance.

Results

Weight response. Because of the reduction in food consumption in the hypobaric pressure chamber pair-feeding of the animals was used. On their first day in the chamber 60 the rats did not eat at all and the average food consumption of the rest was only 3.5 g. No pellets were given to the pair-fed animals during the first 24 h (=24-h fasting). The weight loss during seven days was 15.4% in the rats kept at 40.8 kPa and 14.6% in pair-fed controls. Two out of the 95 rats used died in the hypobaric pressure chamber.

Phosphatidate phosphohydrolase. The activity of this enzyme increased significantly after 24 h in hypoxia, from a control value of 78.3 nmol/min/g wet wt. of liver to 177 nmol/min/g wet wt. ($p < 0.001$). Fasting for 24 h similarly resulted in a significant increase, to 109 nmol/min/g wet wt. ($p < 0.02$) but the difference between the fasting and hypoxic groups was nevertheless highly significant ($p < 0.001$) (Fig. 1). After 7 days in severe hypoxia no differences in the activity of this enzyme could be detected any longer between the *ad libitum* and hypoxic groups, but a higher value was noted in the pair-fed group in comparison with the *ad lib* controls ($p < 0.05$) (Fig. 1).

Triacylglycerol concentration. The increase in the concentration of liver triacylglycerol can be observed in Fig. 1. An increase in phosphatidate phosphohydrolase activity ($p < 0.001$) and liver triacylglycerol concentration ($p < 0.05$) could be observed in the pair-fed group.

ary factors (Hill *et al.* 1958), and therefore the nutritional status of the animals must be carefully controlled in order to reveal the specific effects of other factors.

The hepatic activity of phosphatidate phosphohydrolase, a rate-limiting enzyme in triacylglycerol and triacylglycerol biosynthesis in the liver (Lamb and Fallon 1974 a, b), rises after acute ethanol administration (Savolainen 1977), after subtotal hepatectomy in sham-operated rats (Mangiapane *et al.* 1973). There are no previous reports on its activity during hypoxia.

The present results demonstrate that hepatic phosphatidate phosphohydrolase activity rises in hypoxia, and that there is also an increase in the triacylglycerol concentration. Increase in phosphatidate phosphohydrolase activity was concomitant with an increase in concentration of *sn*-glycerol-3-phosphate. It has been previously shown that hepatic triacylglycerol accumulation correlates with phosphatidate phosphohydrolase activity (Savolainen 1977), and also with hepatic *sn*-glycerol-3-phosphate activity (Savolainen and Haas 1977), and the present results suggest that the same relations exist in hypoxia, although time-course of the changes was not studied in detail. The semi-starvation of the pair normoxic control animals resulted in a slow steady increase in the activity of phosphatidate phosphohydrolase, but only a transitory initial increase in the triacylglycerol concentration. Moreover an increase in phosphatidate phosphohydrolase activity has been observed previously during starvation (Vavročka *et al.* 1969), under conditions in which there is no increase in hepatic triacylglycerol formation (Vavročka *et al.* 1969 Park *et al.* 1972). No explanation is at present available for the behaviour of phosphatidate phosphohydrolase during starvation.

The possibility that peripheral lipolysis may induce an accumulation of triacylglycerols in the liver in hypoxia cannot be excluded, for catecholamine liberation also increases in hypoxia (Cannon and Hoskins 1911 Maher *et al.* 1975). In the present study a significant rise in the plasma free fatty acid concentration was observed in acute hypoxia after 2 h, but the concentration was significantly higher still in the 24-h fasting animals. This probably means that fatty acid liberation from the adipose tissue in hypoxia is at least partly due to the state of semistarvation. The smaller increase in the plasma free fatty acid concentration in the hypoxic animals may indicate that hypoxia itself has some inhibitory effect on lipolysis, an effect that has been observed in perfused rat hearts at least (Criss and Cooper 1975). In the present case the increased lipolysis was not reflected in the hepatic triacylglycerol concentration, which did not differ significantly from the controls in the 24-h group of 7-day pair-fed groups.

The rate of hepatic fatty acid synthesis increased somewhat in acute hypoxia, but returned to normal values during one week of hypoxia. Under the latter conditions, however the hepatic triacylglycerol concentration was more than twice that in the pair-fed controls. On the basis of the present results it is not possible to exclude the possible contribution of an increased fatty acid synthesis rate in the triacylglycerol accumulation, but it seems that during the prolonged hypoxia of seven days the triacylglycerol accumulation is not regulated by the rate of fatty acid synthesis. A positive correlation has previously been observed between hepatic lipogenesis and glycogen concentration (Salmon *et al.* 1974), but this could not be confirmed in the present work on hypoxia. Lactate has also been implicated in lipo-

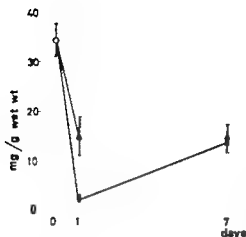


Fig. 4. Effect of hypoxia on hepatic glycogen concentrations. Values are means \pm S.E. from 4-5 separate experiments. Experimental conditions and symbols as in Fig. 1.

Other metabolites The hepatic *sn*-glycerol 3-phosphate concentration increased significantly in acute hypoxia ($p < 0.001$) from a control value of $0.367 \mu\text{mol/g wet wt. of liver}$ to $2.84 \mu\text{mol/g}$ after 30 min under $8\% \text{ O}_2$. The concentration was smaller ($p < 0.1$) at 24 h than after 30 min in hypoxia but was still significantly elevated ($p < 0.001$) compared to the controls. After seven days the concentration was $2.46 \mu\text{mol/g}$, a value higher than that of the controls ($p < 0.001$) and also higher than the value after 24 hours ($p < 0.01$) (Fig. 3). Liver lactate concentration increased in acute hypoxia after 30 min, from a control value of $1.85 \mu\text{mol/g wet wt. of liver}$ to $9.42 \mu\text{mol/g}$ ($p < 0.001$). After 7 days hypoxia the concentration was $5.11 \mu\text{mol/g}$, higher than the control value ($p < 0.001$) but lower than the concentration in the non-adapted animals after 30 min of hypoxia ($p < 0.02$) (Fig. 3). The liver glycogen concentration decreased significantly during the first 24 h in hypoxia ($p < 0.01$), and was still low after seven days in hypoxia ($p < 0.01$) (Fig. 4). Still lower glycogen concentrations ($p < 0.001$) were observed in the 24-h fasting control group (Fig. 4). Blood glucose did not change in hypoxia after either 24 h or 7 days when compared with the *ad libitum* controls, the values being $7.00 \pm 0.08 \text{ mmol/l}$ (mean \pm S.D., $n = 4$) in the control group, $9.40 \pm 0.15 \text{ mmol/l}$ (mean \pm S.D., $n = 4$) after 24 hours at 40.8 kPa and $8.41 \pm 1.22 \text{ mmol/l}$ (mean \pm S.D., $n = 4$) after seven days at 40.8 kPa . The plasma free fatty acid concentration was significantly ($p < 0.05$) higher after 24 h in hypoxia, i.e. $0.54 \pm 0.10 \text{ mmol/l}$ (mean \pm S.D., $n = 4$), as compared with the *ad libitum* controls ($0.36 \pm 0.16 \text{ mmol/l}$, mean \pm S.D., $n = 8$), but not after 7 days. The plasma free fatty acid concentration was similarly significantly higher in the fasting animals ($0.70 \pm 0.13 \text{ mmol/l}$, mean \pm S.D., $n = 8$) after 24 h than in the fed controls ($p < 0.01$) and the hypoxic group ($p < 0.05$).

Discussion

The present results confirm the recently reported increase in hepatic triacylglycerol concentration in hypoxic hypoxia (Kinnula and Hassinen 1978), although the increase was much smaller possibly due to the different rat strain used. Strain differences are also indicated by the lower mortality of the Sprague-Dawley rats in hypoxia. Lipid synthesis is affected

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genesis and it has been suggested that II may have a regulatory role not connected with precursor nature (Salmon *et al.* 1974). No correlation was found between the lactate concentration and the rate of lipogenesis in the present study.

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acetylcholine (ACh) (Helwall *et al.* 1975) and choline acetyl transferase (CAT) (Jedlicka *et al.* 1975) in the sciatic nerve was blocked by COL or VIN. Both these drugs are with cytoplasmic microtubules and bind to the tubule protein *in vitro*. (e.g. Borisy *et al.* 1967; Mahewiza, Sato and Benach 1968). Since the effect of mitotic inhibitors on was paralleled in some neuron systems by a decrease in the number of axonal microtubules (Banks *et al.* 1971, Fink *et al.* 1973), the anti-transport effect was considered due to a specific destruction of the microtubules.

Even both COL and VIN have biochemical effect on cytoplasmic organelles and not other than microtubules and their subunit, tubulin (e.g. Felt and Barondes 1970, Fink *et al.* 1970). Therefore the effects of COL and VIN on AXT may be due to other reasons in the cell and not directly related to their binding to microtubules.

To test whether inhibition of AXT by mitotic inhibitors is related to their effect on microtubules, isomers to mitotic inhibitors, having a documented less effect on microtubules, have been tested. Thus, the isomer to COL, lumenicol (lumCOL) has a much less binding to microtubules than COL (Bryan 1972) and has also a much less effect on the AXT of labelled proteins (Bryan and McClure 1974, 1975). Another pair of substances is podofyllotoxin (POD) and its isomer picropodofyllin (picPOD). POD is an efficient inhibitor of AXT and binds to tubulin like COL (Wilson 1970, Paulson and McClure 1975a, Banks and Tull 1975). The isomer picPOD has less binding to tubulin than POD and also a much less antitransport effect (Banks and McClure 1975 b).

These two pairs of substances, COL and lumCOL on the one hand, and POD and picPOD on the other hand, have now been used to study the involvement of axonal microtubules in the previously demonstrated anterograde AXT of ACh, AChE and CAT in rat motor nerves. A preliminary report of these studies has been published (Dahlgren *et al.* 1975).

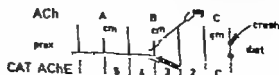
Materials and methods

Animals. For the COL-lumCOL experiments male rats of the hooded strain (250–300 g) were used. Male Sprague-Dawley rats (250–300 g) were used in the other experiments.

Surgical procedure. Under ether anaesthesia the sciatic nerves bilaterally were exposed about 10 mm proximal to the nerve plexus. Under the control of a dissection microscope the test substances were injected suboperatively with 32 gauge needle. The volume of each injection was 3–5 µl, each spread 3–5 mm along the nerve. COL or POD and their respective isomers (lumCOL and picPOD) were tested in pairs, but the test substances were injected into one nerve and the isomer into the contralateral nerve (after 10 min) of the same animal, in equimolar concentrations. 2–3 h after the injection, the animals were re-anaesthetized and a crush operation (see Leharzka 1959) was performed 15 mm distal to the injection level (Fig. 1). After 12–15 h the rats were made unconscious by a blow on the head (for ACh expts.), or decapitated (for enzyme assays), the nerves were dissected out and chilled on an ice-cooled glass-plate prior to assay.

Chemicals. COL (Sigma) was used to prepare lumCOL according to Wilson and Fredrick (1966). 1 M solution of COL in 95% ethanol was irradiated for 36 h with 366 nm UV-light from HPW 125 W

1. Schematic illustration of action level, crush site 15 mm distally and nerve segments used for ACh (A-C) and CAT or AChE (3-5).



Further evidence for the involvement of microtubules in the proximo-distal intra axonal transport of acetylcholine and related enzymes in rat sciatic nerve

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Abstract

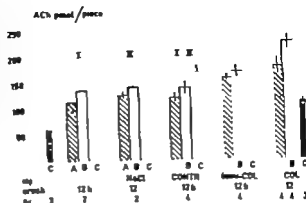
HEIWall, P.-O., P. A. LARSSON and A. DAHLSTRÖM. Further evidence for the involvement of microtubules in the proximo-distal intra-axonal transport of acetylcholine and related enzymes in rat sciatic nerves. *Acta physiol. scand.* 1978. 104. 156-166.

The two mitotic inhibitors colchicine (COL) and podophyllotoxin (POD) and their respective isomers lumicolchicine (lumCOL) and picropodophyllin (picPOD) were tested for their effect on the intra-axonal transport (AXT) of acetylcholine (ACh) and the cholinergic enzymes in rat sciatic nerve. The mitotic inhibitors and their isomers were dissolved in saline + 10% ethanol (COL and lumCOL) or dimethylformamide (DMFA) (POD and picPOD) and injected (3-5 μ l) subcutaneously into the sciatic nerve. As controls the sciatic nerve was crushed distal to the site of injection. The accumulation of ACh or the two enzymes, ACh-esterase (AChE) and cholineacetyltransferase (CAT), in the nerve segment proximal to the crush (12 h before death) was used as a measure of the AXT. COL and POD were very effective in inhibiting AXT of all 3 substances, while the isomers, lumCOL and picPOD, were essentially without effect on AXT in equimolar concentrations. The effects on AXT of the 4 test substances thus appear related to their affinity to bind to tubulin, but several orders of magnitude higher for COL and POD than for their isomers. The results further support the view that intact microtubules are essential for AXT of both membrane-bound (AChE) and soluble (CAT) enzymes, as well as of ACh in rat motor nerves.

Key words. Intra-axonal transport, cholinergic enzymes, acetylcholine, mitotic inhibitors, isomers, tubulin affinity.

The intra-axonal transport (AXT) of proteins, organelles, enzymes and certain transmitters has for many years been suggested to depend on intact microtubules in the axon (for review see Dahlström 1971, McClure 1972, Heslop 1973, Hanson and Edström 1978). This theory is supported by the early experiments with local application of colchicine (COL) or vinblastine (VIN) on adrenergic nerves and sciatic nerve to study the AXT of noradrenaline (NA) granules and acetylcholinesterase (AChE) (Dahlström 1968, Kreutzberg 1969, Banks et al. 1971). The proximo-distal AXT of both these substances was blocked by COL or VIN. Later it was shown that also the anterograde transport of isotope-labelled proteins (e.g. Jørgensen

The ACh content of 12 h old rats active after the neural injection, 2 h before of saline (I), ethanol (II), 10^{-4} M haniCOL (III) or COL (IV). Various segments as indicated in Fig. 1. Individual or mean \pm S.E. are given. $p < 0.05$ and $p < 0.01$ and $p < 0.005$ respectively.



I (group I, Fig. 3). The A and B segments in this group contained 116–137 pmol/10 mm h is a normal amount for 10 mm segments at that level (see also Fig. 6). In nerves injected with the vehicle for the test substances, (physiological saline with 10% of ethanol) and killed 12 h before death (group II, Fig. 2), the ACh-accumulation in segment C was similar to that in group I. No change in ACh-content of segments A and B was caused by vehicle administration (compare groups I and II, Fig. 2). Since the 2 groups were very similar in ACh content, they were taken together as a control group (I + II in Fig. 2). The injection of 0.1 M haniCOL caused a small increase in both segments A and B (significant p for segment A), but the ACh-accumulation in segment C was similar to that in the control group. The injection of 0.1 M COL, on the other hand, caused a marked increase in ACh content in segments A and B, and a significant decrease in the ACh-accumulation in segment C 12 h after crushing (to 110 ± 6 pmol, $p < 0.005$ compared to haniCOL).

AChE. The results in Fig. 4 are expressed in per cent of the enzyme activities in segments 2 and 3 of the saline-treated group in each separate expt. a) because this level of the nerve is crushed (see Fig. 1), and b) because the AChE-accumulation does not reach segment 2 until 13 h after crushing. The results in Fig. 4 are based on 4 expts., and the 100%-value varied between 3457–8640 pmol of ACh split/min/5 mm nerve in the various expts. When taken together $100 \pm 9\%$ corresponded to 6234 ± 578 pmol of ACh split/min/5 mm ($n = 18$). The injection of the vehicle between the segments 3 and 4 (see Fig. 1) did not induce any noticeable change in AChE-activity in the segments 3–5 as compared to a normal uncrushed static nerve. In the C-segment, the enzyme activity was increased to $212 \pm 18\%$ at 13 h after crushing. In the haniCOL-treated group, the enzyme activity in segment C was $166 \pm 20\%$. Thus, it appeared somewhat lower than in the saline group but the difference was non-significant. Following COL-treatment, the AChE activities of segments 4 and 5 were clearly increased as compared to the two other groups, and the activity in segment C was only $10 \pm 17\%$. Thus, no accumulation of enzyme activity appeared to have occurred during the 1 h following crushing (significantly different from the C-segment in the haniCOL-group at $p < 0.025$).

CAT. The results in Fig. 5 are based on 3 different expts. and expressed in per cent of the activity in segments 2 and 3 of the control (saline) group for reasons indicated above.

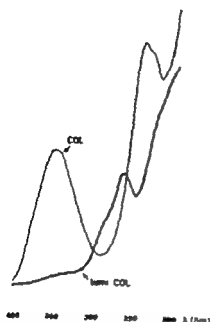
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Fig. 2. The UV absorbance spectra between 200–400 nm of COL and turni-COL, after irradiation at 366 nm.

mercury lamp (Philips). To control that no COL was left in the solution the ultraviolet absorbance was read between 200–400 nm on a Zeiss spectrophotometer. In parallel a COL solution, treated as it but not irradiated, was also read. Only samples in which the typical absorbance peak of COL at 355 nm disappeared were used (Fig. 2). The ethanol present in the solutions was evaporated, and the level weighed and kept in the cold until use. 0.1 M solution of the two substances were prepared just before injection in saline with 5–10% ethanol. In one group of control animals (Fig. 3–5) the chick alone was injected.

POD (Aldrich Chemical Comp. Inc.) was used to prepare the picPOD according to Wilson and Fink (1967). A solution of 200 mg of POD in 10 ml of methanol and 1 ml of a 7% NH_3 was stirred for 3 h and cooled to yield a flocculent precipitate (picPOD). This was washed twice with 10 ml portions of absolute ethanol and dried *in vacuo*. The determination of the melting point was used to control the purity of the picPOD (mp 221–224°C) prepared from POD (mp. 112–114°C). Prior to injection the picPOD was dissolved in dimethyl formamide (DMFA, Merck) to a final concentration of 0.1 M. In a group of animals the vehicle alone was injected and its effect compared to that of saline (Fig. 6–8).

Assay methods: ACh was assayed on 10 or 5 mm segments of nerve as indicated in Fig. 1. 2–4 pieces were pooled for each assay. Following extraction according to Macintosh and Perry (1950) the samples were assayed on a guinea pig ileum preparation (Blaber and Cuthbert 1961). An antihistamine (Mepyramine) was added to the Krebs solution used in the gut bath. Schild's (1949) four point assay was used to calculate the ACh-activity of the samples. ACh was pharmacologically identified by inactivation with purified AChE (Worthington Biochemical Corp., New Jersey) and by atropine inhibition of the responses of the gut standard and sample. The activity measured was thus ACh-like but will be referred to as ACh in the following.

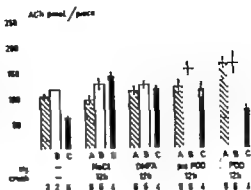
AChE and CAT were assayed in 5 mm segments (Fig. 1) according to the method of Tuček (1970). Both enzymes could be measured in the same nerve segment.

For statistical analysis of the results Student's *t* test was used.

Results

COL-turniCOL experiments

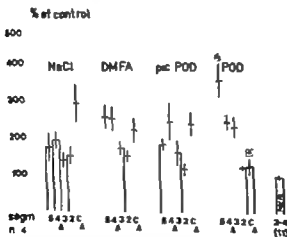
ACh. The ACh-content of a normal uncrushed 5 mm C-segment was 61 ± 1 pmol (Fig. 3). In nerves which were only crushed for 12 h, the content of the C-segment was as



The ACh content of normal, intact and crushed rat sciatic nerve after the subcutaneous injection (3-5 μ l) of saline (NaCl), DMFA, pscPOD (0.1 M) or POD (0.1 M). Various segments as indicated in Mean \pm S.E. are given. *indicate difference from pscPOD group at $p < 0.025$.

In normal, uncrushed sciatic nerve (see Fig. 6), the 5 mm C-segment contained 63 ± 3 pmol of ACh (5). The 10 mm A and B segments contained 104 ± 3 and 117 ± 21 pmol, respectively. In order to compare the effect of solvent used for the test substances (DMFA) the mechanical effect of the injection trauma 2 control groups with 12 h crushes were

AChE



A site of inj 2h before crush

A site of crush 12h before death

Fig. 7 The AChE-activity in rat sciatic nerve after the subcutaneous injection (3-5 μ l) of saline (NaCl), DMFA, pscPOD (0.1 M) or POD (0.1 M). Arrows indicate sites of injection and crush. Mean \pm S.E. given 100 1236 ± 239 pmol of ACh split/min/5 mm nerve.

Indicate different from NaCl at $p = 0.025-0.05$

Indicate different from DMFA at $p = 0.025$

Indicate different from pscPOD at $p = 0.01$ and $p = 0.025$ respectively

AChE

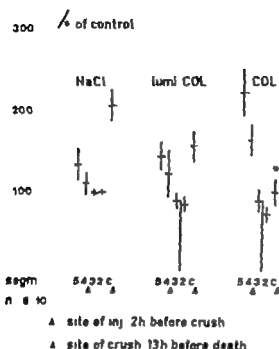


Fig. 4 The AChE-activity in rat sciatic nerve after the subperineural injection (3–5 μ l saline + 10% ethanol (NaCl), lumiCOL (0.1 M) or COL (0.1 M). Arrows indicate site of injection and crush. Mean \pm S.E. are given. * = 6.234 \pm 578 pmol of ACh split/min/5 mm nerve. — indicates difference between lumiCOL and COL groups at $p < 0.05$.

(AChE-section). $100 \pm 2\%$ corresponded to 1.140 ± 27 pmol of ACh formed/min/5 mm segment. In the C-segment of the control group, $110 \pm 5\%$ of CAT-activity was present after crushing while in the lumiCOL group the level was $115 \pm 3\%$ in the same segment. In the COL treated group, however, the C-segment contained only $99 \pm 4\%$ ($p < 0.005$, compared to the lumiCOL group) while segments 4 and 5 contained somewhat higher cat activities than the corresponding segments of the 2 other groups (Fig. 5).

CAT

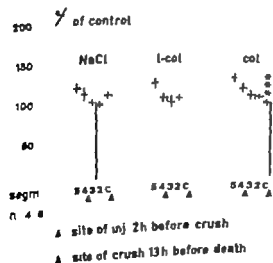
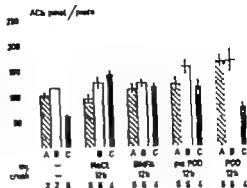


Fig. 5 The CAT-activity in rat sciatic nerve after the subperineural injection of 3–5 μ l saline + 10% ethanol (NaCl), lumiCOL (0.1 M) or COL (0.1 M). Arrows indicate site of injection and crush. Mean \pm S.E. are given. * = 1.140 \pm 27 pmol of ACh formed/min/5 mm nerve. — indicates difference between C-segment of lumiCOL group at $p < 0.05$.

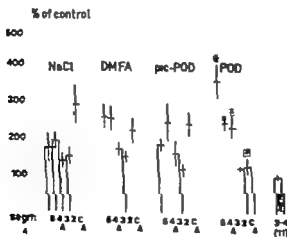


5 The ACh content of normal, intact and crushed rat sciatic nerve after the subneural injection (3-5 μ l) of saline (NaCl), DMFA, pc-POD (0.1 M) or POD (0.1 M). Various segments as indicated in 1. Mean \pm S.E. are given. *Indicate different from pc-POD group at $p < 0.025$.

D-pc-POD experiments

4 In normal, uncrushed sciatic nerve (see Fig. 6), the 5 mm C-segment contained 63 ± 3 μ mol of ACh ($n = 5$). The 10 mm A and B segment contained 104 ± 3 and 117 ± 21 μ mol, respectively. In order to compare the effect of solvent used for the test substances (DMFA) and the mechanical effect of the injection trauma 2 control groups with 12 h crushes were

AChE



A site of my 2h before crush

B site of crush 12h before death

6 The AChE-activity in rat sciatic nerve after the subneural injection (3-5 μ l) of saline (NaCl), DMFA, pc-POD (0.1 M) or POD (0.1 M). Arrow indicates site of injection and crush. Mean \pm S.E. are given. 100 ± 299 μ mol of AChE unit/min/5 mm nerve.

* indicate different from NaCl at $p < 0.025-0.05$

† indicate different from DMFA at $p < 0.025$

‡ indicate different from pc-POD at $p < 0.01$ and $p < 0.025$ respectively

CAT

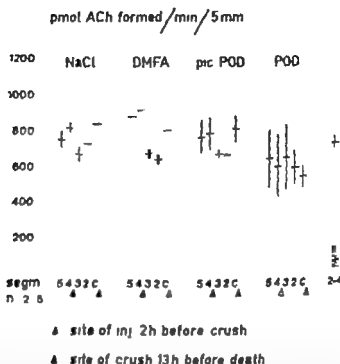


Fig. 8. The CAT-activity in rat sciatic nerve after the subepineurial injection (3–5 μ l) of saline (V-vehicle), DMFA, picPOD (0.1 M) or POD (0.1 M). Arrows indicate sites of injection and crush. Values or mean \pm S.E. are given. Normal 5 mm nerve contains 724–34 pmol of ACh formed/min/5 mm nerve. Δ indicates different from C segment of the picPOD group at $p < 0.05$.

Included one group with DMFA injections in the B-segment and one with saline injection (see Fig. 6). In the saline injected group 143 ± 8 pmol was present in segment C while the segment of the DMFA injected group contained 113 ± 13 pmol of ACh. Thus, a small insignificant decrease in ACh-accumulation had probably occurred after DMFA-treatment. When the test substances were injected into the nerve the C-segment contained 115 ± 13 pmol of ACh in the picPOD-group while in the POD-group only 75 ± 9 pmol was present (no difference between the two test substances at $p = 0.025$). The A and B segments of the POD group contained somewhat higher ACh-content than the same segments of the other groups (difference of the A-segment against the picPOD-group $p = 0.025$).

AChE. The results shown in Fig. 7 represent 2 expts. and are expressed in per cent of crushed uninjected segments (2–5) for each experimental occasion. $100 \pm 5.4\%$ corresponded to 3.36 ± 259 pmol of ACh split/min/5 mm segment. As in the ACh part of this experiment (above), two control groups were included: one injected with saline and the other with DMFA. The DMFA injection appeared to cause a) an increase in segments 4 and 5 (above the injection) as compared to the saline group and b) a decrease in the amount of enzyme activity which had accumulated in segment C during the 12 h of crushing. The differences were not significant, however. In the group injected with picPOD the AChE-activities of the various segments was about the same as in the DMFA-treated group. The POD-group had

τ showed significant differences as compared to the other 3 groups. The AChE present in proximal C was only $127 \pm 22\%$ as compared to 240 ± 28 and $223 \pm 31\%$ in the picPOD and DMFA-group, respectively ($p < 0.025$). In segments 3-5 there was an increased AChE activity particularly in segment 5 ($356 \pm 43\%$) as compared to the picPOD-group or the DMFA-group ($p = 0.01-0.025$).

CAT The results in Fig. 8 represent 2 experiments and are expressed in pmol of ACh formed/min/5 mm nerve. A normal 5 mm segment contained 724 ± 34 pmol of ACh formed/min/5 mm segment ($n = 5$). The control groups, injected with saline or DMFA, showed their similar enzyme activities. The CAT-activity of the C-segments at 12 h after crushing is thus as a mean 842 and 808 pmol of ACh formed/min/5 mm in the 2 groups, respectively (Fig. 8). In the picPOD-treated nerves the CAT-activity of segment C was $811 \pm 75\%$, and the enzyme activities of the other segments was similar to those in the 2 other groups. After DMFA-treatment, however, the CAT-activity of the C-segment was only 540 ± 61 pmol of ACh formed/min/5 mm (different from the picPOD-group at $p < 0.025$). In the other segments, the CAT-activities showed a large variation (Fig. 8).

Discussion

The biological method to assay ACh has been discussed earlier (Dahlstrom *et al.* 1974). The assay performed, i.e. incubation with purified AChE and atropine block of the gut, strongly suggest that the measured activity was ACh. This is also supported by results from chemical termination of ACh in rat sciatic nerve by Ullin *et al.* (1976) which are in excellent agreement with our values.

The radiochemical methods for assaying AChE and CAT in the same homogenate have been discussed by Tuček (1974). The enzyme activities observed in this study agree well with those previously observed by other investigators, except that the AChE activities in some experiments have been unusually high (up to about $100 \mu\text{mol ACh split/h/g}$ of nerve, as compared to $56 \mu\text{mol per h/g}$ of rat sciatic nerve as reported by Kani *et al.* (1973)). The CAT activity varied in this study between $8.5-13 \mu\text{mol of ACh formed/h/g}$, while previous investigators found in rat sciatic nerve a CAT-activity corresponding to $8-10 \mu\text{mol of ACh formed/h/g}$ (Forman 1969; Saunders *et al.* 1973).

As seen in Fig. 3-5 the injection of 0.1 M COL into the sciatic nerves 2 h before the crush operation caused a decreased accumulation of ACh, AChE and CAT in segment C, just above the crush. For all 3 substances this decrease, when compared to the humCOL group, was significant. Since the amount of ACh, AChE and CAT appeared increased around and above the areas of injection of COL, but not (or to a much less extent) of humCOL, the results suggest that COL, but not humCOL, had interrupted the proximo-distal AXT of ACh and the two enzymes, thus reducing the accumulations further distally in the C-segment. HumCOL, a structural isomer of COL, has many biological properties in common with COL, but differs markedly in its affinity to bind to the microtubule subunit protein, tubulin. HumCOL has less than $1/100$ of the affinity of COL to tubulin (Wilson and Friedkin 1967; Wilson *et al.* 1974; cf. also Paulson and McClure 1975). The transport-inhibiting capacity of these two agents thus appear to be correlated to their affinity to tubulin. The fact that

lumiCOL appeared to have a small effect on the accumulation of AChE-activity (Fig. 4) in agreement with the fact that this drug does have a small affinity for tubulin.

The other pair of substances tested, POD and picPOD gave results essentially similar to those discussed above. The vehicle itself DMFA, appeared to interfere to some degree with the transport of especially ACh and AChE, as judged from the small decrease in accumulated amounts in the C-segments (Fig. 6 and 7). However the addition of picPOD (0.1 M) to DMFA did not cause any further disturbances in the transport of ACh and AChE, while POD-injection had very marked effects on both ACh and the enzymes. Above and around the areas of injection of POD both ACh and AChE-activity increased, and in the C-segment the accumulation of all 3 substances was significantly lower than in the picPOD group. Thus picPOD had much less effect on the axonal transport of both ACh and the enzymes than had POD in equimolar concentrations. POD binds 135 times more to tubulin than does picPOD (Wilson and Friedkin 1967 Paulson and McClure 1975). Thus, also in this case there seems to be a correlation between the tubulin affinity of the substances and its transport inhibitory effect.

The above substances were injected in seemingly very high concentrations, if compared with the mM-concentrations used in *in vitro* expts. (Edström and Mattson 1972, Paulson and McClure 1975 Hansson and Edström 1977). However the few μ l of test solution injected into the nerve spread over about 10 mm (cf Dziegielewska *et al* 1975) and probably equilibrated in a volume of at least 10 μ l. Therefore, the tissue concentration of these substances probably was in the order of 25 mM which is less than an order of magnitude higher than the concentration of e.g. COL used *in vitro* to block axonal transport in frog sciatic nerve (5 mM Edström and Hansson 1974).

The results of the present study show that lumiCOL and picPOD had much less inhibitory effect on rapid AXT of AChE and ACh than COL and POD and are in agreement with results by other investigators. Thus, Paulson and McClure (1974, 1975) could arrest rapid AXT of labelled proteins *in vitro* by COL, POD and VIN but had no effect of lumiCOL, or picPOD. In rabbit optic nerves protein transport was inhibited by COL but not by lumiCOL (Paulson 1974). The transport of noradrenaline storage vesicles in cat hypogastric nerves was blocked *in vitro* by COL but not by lumiCOL (Banks and Till 1975). These results strongly suggest that microtubules are involved in rapid AXT of various substances, but do not rule out the possibility that both COL and POD may interfere with AXT by acting on processes not directly related to microtubules.

We have previously reported that both COL and VIN interfere with the transport of ACh (Heiwall *et al* 1975) and CAT (Dziegielewska *et al* 1975) in rat sciatic nerve, suggesting the participation of microtubules in transport of both these substances. This suggestion has been further strengthened in the present study. The interesting thing is that also the transport of the soluble enzyme CAT appears to be dependent on microtubules (see Fig. 5 and 8) in rat sciatic nerve (this enzyme appears to move with the so called slow phase of AXT but a very small fraction may theoretically move faster (cf Saunders *et al* 1973). The dependence on intact microtubules also of this probably slow transport (i.e. a slow rate of mean transport velocity) is in agreement with the "microstream hypothesis" of Gross (1975) or the "transport filament theory" of Ochs (1971).

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Suppression of autonomic postganglionic discharges by pentobarbital in dogs, with or without endotoxemia

By

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Abstract

LINEN, M. O., M. O. K. HAKUMÄKI and H. S. S. SARAJÄS. *Suppression of autonomic postganglionic discharges by pentobarbital in dogs, with or without endotoxemia.* Acta physiol. scand. 1978. 104. 167-174.

al effects of pentobarbital (8 mg/kg) on autonomic efferent and afferent discharge rates were studied in dogs under morphine-chloralose anaesthesia. Half of the dogs were given endotoxin *E. coli* (1 mg/kg) and pentobarbital. The postganglionic cervical vagal efferentiation of all the dogs decreased as did the sympathetic cardiac efferentiation. The heart rate of the dogs given endotoxin decreased, as it increased in heart rate with abolition of respiratory arrhythmia, was observed in dogs without endotoxin. The aortic pressure of the former dogs dropped while it fell only slightly in the latter ones. Aortic arch baroreceptor activity decreased while the changes of left atrial B-type receptor activity were not significant. The changes of the left atrial and central venous pressures were slight but those of the lumbar arterial pressure generally paralleled the changes in the aortic pressure. Pentobarbital, acutely seems to exert both sympatholytic and vagolytic effects. These explain the heart rate changes, as well as the impaired cardiac contractility it evokes. The obvious impairment of cardiovascular control mechanisms by pentobarbital should be seriously considered in investigations into the cardiovascular con-

entobarbital has been, and still is, one of the most widely used anaesthetics in cardiovascular experimentation. However the effects of the drug are generally disregarded in the interpretation of the data obtained. This is due to the fact that the circulatory and the autonomic effects of pentobarbital are not completely known (Manders and Vaiter 1976).

Lacking direct evidence, it has been difficult to differentiate the direct effects of pentobarbital on the heart and blood vessels, from those on the autonomic nervous system which, again, may secondarily modulate cardiovascular functions (Horwitz 1977). Thus, the concept of the vagolytic effect of the drug (Morrison, Walker and Richardson 1960, Vidrio and Pardo 1967, Page and Hoff 1969) emanates from the varying degrees of tachycardia it evokes in experimental animals (Van Citters, Franklin and Rushmer 1964, Gilmore 1965). But under special circumstances, the drug reportedly elicits even bradycardia (Swanson and Shoule 1930). On the other hand, a sympathetic activation by pentobarbital has been suggested from observations on its hypertensive effects (Rushmer, Van Citters and Franklin

1963 Priano Traber and Wilson 1969 Sawyer Lumb and Stone 1971 Golding *et al* 1971). In dissonance with this are findings of decreased vascular smooth muscle (Harvey 1975), of decreased myocardial contractility and of impaired left ventricular function following injections of pentobarbital (Vatner and Braunwald 1975). The cardiac depression in response to barbiturates is greatly enhanced in states of circulatory shock (Rosen 1943 Becher 1951 Van Bergen 1963 Enderby 1965 and Wolfson 1966). Apart from the cardiac actions (Cox 1972 *a*), a pentobarbital-induced sympatholysis may be important in effecting this myocardial depression.

In order to gain insight into the conflicting data just outlined, we have studied the barbiturate-induced changes in the spontaneous discharge rates of autonomic nerve fibers controlling the heart and circulation in dogs under morphine-chloralose anesthesia. Peripheral responses of the heart rate and blood pressures were also recorded. To upset the compensatory status and reflex functions, half of the dogs were given endotoxin prior to administration of the barbiturate. Thus experiments were performed on two groups of dogs, a control group being in as normal condition as the method warrants and an endotoxic group being in a state of shock induced within a few minutes.

Material and methods

The experiments were carried out on 26 mongrel dogs, weighing from 7 to 29 kg. Following premedication with subcutaneous morphine hydrochloride (1 mg/kg) the dogs were anesthetized with α -chloralose (100 mg/kg) dissolved in warm physiological saline. After the induction of anesthesia animals were intubated, and the tracheal tubing was connected to a volume controlled respirator. The thoracic aorta and the inferior caval vein were cannulated via the femoral vessels. Arterial blood samples were taken anaerobically during the operative phase for acid-base and blood gas determinations. Possible shifts in these were corrected to physiological limits, by adjusting the pulmonary ventilation by i.v. administration of sodium bicarbonate solution. This was considered mandatory because the responsiveness of the cardiovascular system to catecholamines is depressed by acidosis (Darby *et al* 1965) and because changes in the arterial carbon dioxide tension modify the sympathetic tone (Mäkelä and Kivimäki 1965).

A left thoracotomy was performed and the upper lobe of the left lung was resected. The pulmonary artery and left atrium were cannulated via the vessels of the resected lobe. All pressures were recorded with strain gauge transducers (Hewlett Packard 267BC) and amplified with a carrier amplifier (Hewlett Packard 8405B).

In the thorax, a mineral oil pool was created from the parietal pleura. The sympathetic postganglionic discharges were recorded from the fibers of the third cardiac nerve. On the neck the left vagus nerve was exposed and cervical efferent vagal impulses with cardiac rhythm were recorded. The aortic arch receptor and the left atrial B-type receptor fiber activity were also recorded on the neck.

One half of the dogs (13) were given intravenous endotoxin *Escherichia coli* 1 mg/kg 3–15 min before recordings were started.

Pentobarbital (Nembutal® Abbott) was given to the dogs given endotoxin (endotoxic dogs) and to the dogs without the toxin (control dogs). The dose levels were 7–9 mg/kg (mean 8.1 ± 1.1 S.D.), and the drug was injected within 15 s. All the neural and circulatory variables were recorded on magnetic tape on a instrumentation tape recorder (FI 6200, Precision Instrument, U.S.A.). The recordings were started 1–2 min before the pentobarbital injection and continued for a total of 3 runs 20 s. Thereafter recordings lasting 20 s, were started at 5, 9 and 15 min.

The action potentials recorded were calculated with a multichannel analyzer (Nokia LP 4340, Nokia Electronics, Finland). The means of relative changes (per cent of control levels) in all recordings of the same fiber type were calculated for each 20 s period. The t-test was used to test the significance of changes and of the differences between the two groups of dogs.

The methods used are detailed elsewhere (Halinen 1976).

1. Mean control values of heart rate and blood pressures. The means of control period heart rates (HR), systolic (AP syst) and diastolic aortic pressures (AP diast), and systolic (PAP syst) and diastolic pulmonary arterial pressures (PAP diast) in dogs under morphine-chloralose anaesthesia (control dogs) and in dogs under morphine-chloralose anaesthesia given endotoxin (endotoxic dogs). Statistical significance of differences between the two groups of dogs is given in the table.

	HR beats/min	AP _{syst} mmHg	AP _{diast} mmHg	PAP _{syst} mmHg	PAP _{diast} mmHg
control	115 ± 52 0.001	142 ± 14 0.001	102 ± 11 0.001	25.1 ± 4.1 0.20	15.0 ± 3.7 0.01
endotox	202 ± 43	80 ± 21	62 ± 20	31.1 ± 6.7	26.7 ± 7.0

Results

Generally, the control heart rates and pulmonary arterial pressures in the endotoxic dogs exceeded those in the control dogs, while the reverse held true for the aortic pressures (ble 1).

The endotoxic dogs were acidotic (pH_a 7.265 ± 0.081 mean \pm S.D.) as compared with control dogs (pH_a 7.373 ± 0.042 $p < 0.001$). The plasma bicarbonate of the former dogs was 3 ± 2.8 mmol/l and that of the latter ones 20.8 ± 2.5 mmol/l, ($p < 0.001$). The mean arterial red oxygen tension of the endotoxic dogs (80 ± 23 mmHg) was lower ($p < 0.001$) than that of control dogs (132 ± 32 mmHg). The mean arterial carbon dioxide tension of both groups was about 30 mmHg.

The responses to pentobarbital in a typical dog are illustrated in Fig. 1. There was aortic pressure oscillation and respiratory arrhythmia, which faded off soon after pentobarbital injection. Along with its stabilization, the heart rate mounted to a higher level. Concurrently the vagal cardiac efferentation showed a more marked suppression than did the sympathetic one. The average baroreceptor activity scarcely changed, but following pentobarbital, as the aortic pressure and heart rate were stabilized, the fluctuation of the baroreceptor impulse activity diminished.

Control dogs. Fig. 2 summarizes the changes in the cardiovascular variables and nerve discharge rates following the pentobarbital injection. The mean heart rate (13 dogs) increased from 115/min to 149/min ($p < 0.05$), 1 min after the pentobarbital injection. Following subsequent cardiac deceleration, equally increased heart rates were seen at 9 and 15 min. Concurrently the mean aortic pressure (13 dogs) fell from 142/102 mmHg to 127/94 mmHg ($p < 0.05$). The pulmonary arterial pressures showed a slight initial increase ($p < 0.05$) from 25/15 mmHg but then showed a trend to decrease.

The cervical vagal efferentation (8 dogs; Fig. 2a and 3) was decreased 30% after pentobarbital injection. It decreased to a minimum of 60 per cent of the control level ($p < 0.01$) at 3 min after the injection. Later on, the vagal efferentation recovered towards the control level (Fig. 2).

1963 Priano Traber and Wilson 1969 Sawyer Lumb and Stone 1971 Golding *et al.* In dissonance with this are findings of decreased vascular smooth muscle response (Harvey 1975), of decreased myocardial contractility and of impaired left ventricular function following injections of pentobarbital (Vatner and Braunwald 1975). The cardiac action in response to barbiturates is greatly enhanced in states of circulatory shock (E. 1943 Becher 1951 Van Bergen 1965, Enderby 1965 and Wolfson 1966). Apart from cardiac actions (Cox 1972 a), a pentobarbital-induced sympatholysis may be involved in effecting this myocardial depression.

In order to gain insight into the conflicting data just outlined, we have studied barbiturate-induced changes in the spontaneous discharge rates of autonomic nerve controlling the heart and circulation in dogs under morphine-chloralose anesthesia. The responses of the heart rate and blood pressures were also recorded. To upset the normal status and reflex functions, half of the dogs were given endotoxin prior to administration of the barbiturate. Thus experiments were performed on two groups of dogs; a group being in as normal condition as the method warrants and an endotoxic group in a state of shock induced within a few minutes.

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A left thoracotomy was performed and the upper lobe of the left lung was resected. The pulmonary and left atrium were cannulated in the cavities of the resected lobe. All pressures were recorded strain gauge transducers (Hewlett Packard 678C) and amplified with a carrier amplifier (Hewlett Packard 8305B).

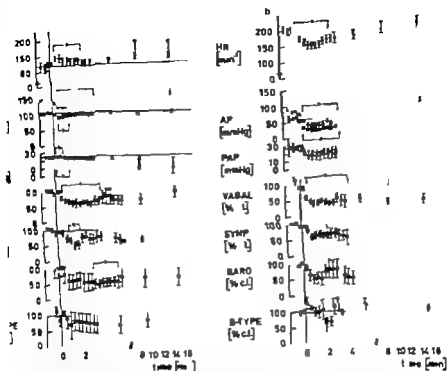
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The action potentials recorded were calculated with multichannel analyser (Nokia LP 4340, Nokia Electronics, Finland). The means of relative changes (per cent of control levels) in all recordings of the same fiber type were calculated for each 20 s period. The t-test was used to test the significance of changes and of the differences between the two groups of dogs.

The methods used are detailed elsewhere (Halinen 1976).



2 a. Effects of pentobarbital (8 mg/kg) given at zero time on dogs under morphine-chloralose anesthesia (seven dogs). HR = heart rate; AP = systolic and diastolic aortic pressures; PAP = systolic and diastolic pulmonary artery pressures; VAGAL = cervical vagal afferent discharges; BARO = aortic arch baroreceptor discharges; B-TYPE = left atrial B-type receptor discharges; % c.f. = mean baroprobe frequencies as per cent of control level frequency. The vertical bars indicate ± 1 S.E. and the horizontal lines the control of each parameter. Statistical significance ($p < 0.05$) is labeled with asterisks.

2 b. Effects of pentobarbital (8 mg/kg) injected at zero time on dogs under morphine-chloralose anesthesia and endotoxaemia (endotoxin 2.0 mg/kg; endotoxin group). For abbreviations see Fig. 2 a.

0.001) and H was attained at 1.3 min. The vagal discharges showed no recovery in the run-up period.

The cardiac sympathetic efferentation (3 dogs, Fig. 2 b) decreased during the cardiac elevation to minimum of 55 per cent of the control level ($p < 0.05$), at about 2 min.

The aortic baroreceptor activity (5 dogs, Fig. 2 b) decreased with the falling aortic pressure ($p < 0.05$). The nadir of 83 per cent occurred at 2 min.

The mean left atrial B-type receptor discharge rate (7 dogs, Fig. 2 b) showed considerable variation following pentobarbital injection. The minimum discharge rates were encountered at 2 min, but at the end of the experimental period their firing was at the control level.

Discussion

Lacking direct instrumentation for recordings of autonomic discharges in conscious animals, general anesthesia was used in the present experiments. The premedication dose of morphine, even small, may augment the vagal tone (Page and Hoff 1969). Chloralose was selected for

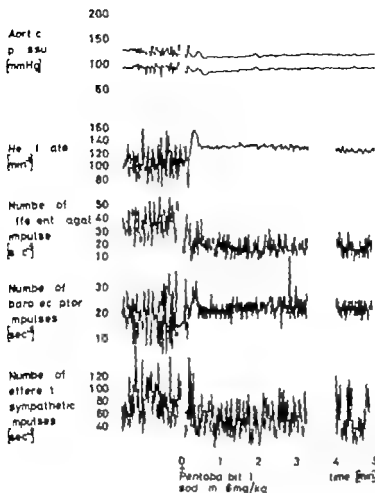


Fig. 1 Circulatory and nervous responses of a dog to morphine-chloralose anaesthesia and pentobarbital injection (150 mg/kg).

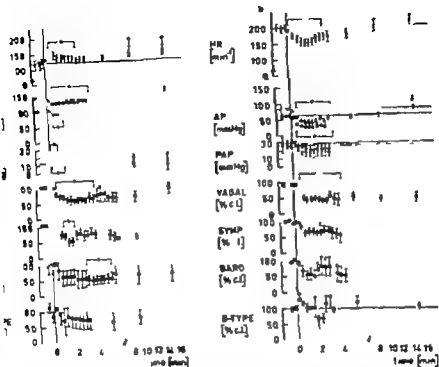
The cardiac sympathetic efferentation (5 dogs; Fig. 2a and 4) decreased significantly ($p < 0.05$) 1–2 min after the pentobarbital injection. During its maximum reduction, the average discharge rate was 52 per cent of the control level (Fig. 2a).

The aortic baroreceptor activity (5 dogs; 2a) decreased concomitantly with the fall in aortic pressure. At 4 min, the baroreceptor activity was 51 per cent of the control level ($p < 0.05$).

The mean left atrial B-type receptor discharge rate (3 dogs; 2a) showed a trend to decrease ($N.S.$) after the pentobarbital injection.

Endotoxin dogs The heart rate (13 dogs; Fig. 2b) decreased from 202/min to 154/min ($p < 0.001$) following pentobarbital. The lowest values occurred at 1–2 min. At 15 min the heart rate had accelerated to 217/min. The mean aortic pressure (13 dogs) initially dropped from 80/62 mmHg to 43/30 mmHg in this group of dogs. The decrease was highly significant ($p < 0.001$). Subsequently the mean aortic pressure mounted to above the control level. The mean pulmonary arterial pressure decreased from 31/27 mmHg to 25/19 mmHg ($N.S.$) after the pentobarbital injection (4 dogs; Fig. 2b).

The cervical vagal efferentation (8 dogs; Fig. 2b) began to decrease 30 s after the pentobarbital injection. The minimum average discharge rate was 41 per cent of the control



a. Effects of pentobarbital (8 mg/kg) given at zero time on dogs under morphine-chloralose anesthesia (1 group). HR: heart rate; AP: systolic and diastolic aortic pressures; PAP: systolic and diastolic pulmonary arterial pressures; VAGAL: cervical vagal efferent discharges; BARO: aortic arch baroreceptor discharges; B-type: left atrial B-type receptor discharges. \pm 1 S.E. mean frequency frequencies as % of control level frequency. The vertical bars indicate \pm 1 S.E. and the horizontal lines the control of each parameter. Statistical significance ($p < 0.05$) is labeled with asterisk.

b. Effects of pentobarbital (8 mg/kg) injected at zero time on dogs under morphine-chloralose anesthesia and endotoxemia (endotoxin 1 mg/kg; endotoxin group). For abbreviations see Fig. 2 a.

0.001) and it was attained at 1–3 min. The vagal discharges showed no recovery in the run-up period.

The cardiac sympathetic efferentation (3 dogs, Fig. 2 b) decreased during the cardiac elevation to a minimum of 55 per cent of the control level ($p < 0.05$), at about 2 min.

The aortic baroreceptor activity (5 dogs; Fig. 2 b) decreased with the falling aortic pressure ($p < 0.05$). The nadir of 53 per cent occurred at 2 min.

The mean left atrial B-type receptor discharge rate (7 dogs, Fig. 2 b) showed considerable variation following pentobarbital injection. The minimum discharge rates were encountered at 2 min, but at the end of the experimental period their firing was at the control level.

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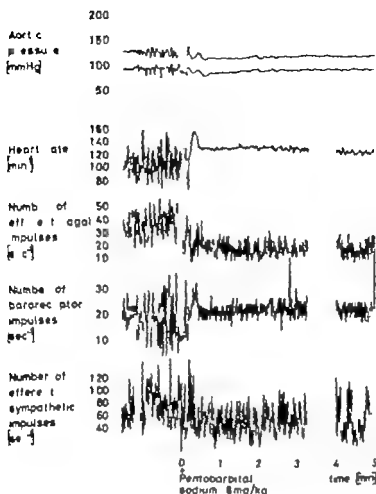


Fig. 1. Circulatory and autonomic responses of a dog to morphine-chloralose anaesthesia and pentobarbital injection (5 mg/kg).

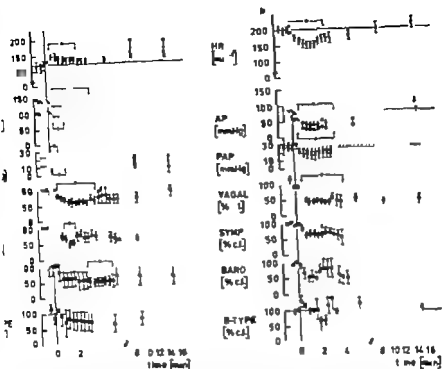
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2 b. Effects of pentobarbital (8 mg/kg) injected at zero time on dogs under morphine-chloralose anesthesia and endotoxaemia (endotoxin $E. coli$ 1 mg/kg; endotoxic group). For abbreviations see Fig. 2.

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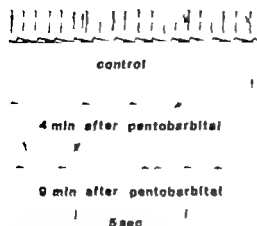


Fig. 3

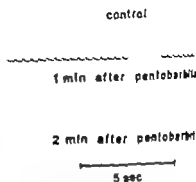


Fig. 4

Fig. 3. Action potentials recorded from an efferent cervical vagal nerve fiber and the aortic pressure before and after pentobarbital injection in a dog under morphine-chloralose anesthesia (control). The horizontal lines indicate the aortic pressure levels of 200, 100 and 0 mmHg.

Fig. 4. Discharges in an efferent cardiac sympathetic fiber and the aortic pressure curve before and after pentobarbital injection in a dog under morphine-chloralose anesthesia and endotoxin (endotoxin 1 mg/kg). The horizontal lines indicate the aortic pressure levels of 100 and 0 mmHg.

basic anesthesia because it leaves the autonomic balance and reflexes largely intact (1960, Greisheimer 1963, Cox 1972 b). Admittedly the possibility exists that the observed responses reflect an interaction of pentobarbital and morphine-chloralose anesthesia. However, the heart rate and blood pressure responses to pentobarbital (tachycardia and hypotension) were similar to those it usually elicits in dogs without underlying morphine-chloralose anesthesia (Cox 1972 a). With these statements in mind we believe our results warrant the following facets of evaluation.

Control state. The slight tachycardia in the control group of dogs was probably a result of an activation of the sympathetic nervous system, by the surgical trauma inherent in the preparative procedures. The endotoxic dogs were in a state of shock as judged from their basic heart rate, arterial pressure and acid base balance (Ertlinger and Suter 1970, Ledsa, Linden and Norman 1971).

Pentobarbital effects. The small pentobarbital dose (8 mg/kg) injected, immediately depressed both the vagal and the sympathetic efferentation. The mean heart rate in the control dogs increased while a decrease in heart rate occurred in the endotoxic dogs. Like the heart rate changes in these two groups, the arterial pressure changes differed as well. In the control dogs a slight, though significant, fall was observed, whereas a marked drop occurred in the endotoxic dogs. The pulmonary arterial pressure of the former group slightly rose, while it fell in the latter group. The aortic arch baroreceptor activity decreased equally in both groups. Concomitantly the sympathetic efferentation was reduced and the respiratory arrhythmia was abolished. This set of events suggests depression of the baroreceptor reflex. No significant changes were found in the atrial B-type receptor discharges and the left atrial mean pressure.

The observed decrease of the vagal efferentation accounts for the tachycardia finding with pentobarbital anesthesia. This substantiates the earlier finding

vagolytic action of the drug (*cf* Introduction). The existing evidence regarding the atibetic responses to pentobarbital is also indirect but conflicting. The suppression of cardiac sympathetic tone observed following pentobarbital in the present experiments may be more apparent than real. It possibly resulted from a reduction of antecedent sympathetic activation by anaesthesia and surgical trauma. Yet, this sympathetic suppression masks the bradycardic reactions occasionally observed in response to pentobarbital (Anson and Shonk 1930, Cox 1972 a). It also explains the impairment of cardiac conductivity and left ventricular functions by pentobarbital (Cox 1972 a, Vatner and Braunwald 1975, Manders and Vatner 1976). The myocardial depression may be reinforced by direct effects of pentobarbital on the heart. It is interesting to note, however that iv thiopental does not affect heart rate, arterial pressure, and cardiac contractility like pentobarbital, but thiopental given directly into the left circumflex coronary artery has no demonstrable inotropic depressant action (Horowitz 1977). In addition, the possible sympathetic suppression effected with the induction of anaesthesia, could be responsible for the greatly enhanced uterine depressant effects of barbiturates in oligoemia (Halford 1943, Becher 1951, d. Bergen 1965, Wollson 1966) and for the precipitation of cardiac arrest by these agents (Cox 1965). The sympatholytic origin of this phenomenon, however remains obscure as outlined above.

The present results are compatible with the concept on the dominant role of the vagal tone in controlling the heart rate in normal circumstances, while in the presence of heart failure the adrenergic component is more operative (Braunwald 1974). Also in endotoxin shock, the sympathetic tone excites the heart more than the increased vagal tone suppresses (Hakinen 1976). However the methods used do not warrant quantifying the relationship between changes in efferent autonomic activity and changes in heart rate.

Concluding remarks. Subanaesthetic doses of pentobarbital, accordingly have both sympatholytic and vagolytic effects. These explain the heart rate changes by the drug and the decrease in cardiac contractility as well. The baroreceptor reflexes, too, are apparently depressed by pentobarbital. Thus, pentobarbital seems to impair the cardiovascular control mechanisms. This explains the susceptibility to barbiturates of patients in circulatory shock.

Obviously the results from experimentation on circulatory physiology in animals with barbiturate anaesthesia must be interpreted with extreme care. The same holds for extrapolations of such experimental data in clinical situation.

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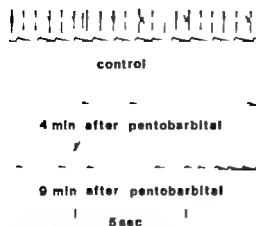


Fig. 3

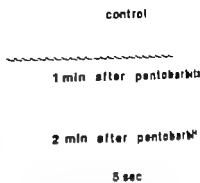


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Thyrotropin-releasing factor Distribution in neural and gastrointestinal tissues

By

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Abstract

LEPPÄLUOTO, J., F. KORVUHALO and R. KRAAMA. *Thyrotropin-releasing factor Distribution in neural and gastrointestinal tissues.* Acta physiol. scand. 1978. 104. 175-179

Concentrations of several tissues were purified by methanol extraction, gel filtration and cation exchange chromatography and measured for thyrotropin-releasing factor (TRF) concentration by radioimmunoassay. TRF immunoreactivity of neural tissues (hypothalamus, spinal cord, medulla, cerebrum and cerebellum) and gastrointestinal tissues (pancreas, stomach, duodenum and colon) behaved in synthetic TRF using the parabiosis. The dual distribution suggests that TRF-secreting cells have their origin in the neural neuroectoderm.

• *Key words:* TRF distribution, gastrointestinal, neural

Thyrotropin-releasing and release inhibiting factors or hormones are a group of small polypeptides which regulate the release of anterior pituitary hormones. Thyrotropin-releasing factor (TRF) was first isolated and characterized as pGlu-His-Pro-NH₂ from ovine (Burgess *et al.* 1969) and porcine (Nair *et al.* 1970) hypothalamus. Later studies have demonstrated presence of a TRF-like peptide by radioimmunoassay in extracts of other brain areas and spinal cord, and in an immunocytochemical study TRF-positive material was located in spinal cord (Jackson and Reichlin 1974, Oliver *et al.* 1974, Hokfelt *et al.* 1975, Kordon *et al.* 1977). It has been postulated that all peptide hormone-producing cells are derivatives of the embryonal neuroectoderm and hence distributed in adults in the central nervous system, gastrointestinal tissues and skin (Pearse 1976). This postulate should explain why substance P, somatostatin, gastrin, vasoactive intestinal peptide and neurotensin-like activity have been found both in the brain and gastrointestinal tract. In this report we have obtained chemical evidence that TRF-like peptide is located in various gastrointestinal tissues, and confirm our previous preliminary results (Leppäluoto *et al.* 1977).

Material and methods

Male Sprague-Dawley rats weighing 200-300 g were used. After decapitation specimens of following tissues (weighing ca. 1 mg-1 g) were removed: hypothalamus, pituitary gland and medulla, spinal

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1. Chromatographic characteristics of TRF immunoreactivity in purified rat hypothalamic and pancreatic samples. Results are means of 2-3 separate thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) runs. The following solvent systems were used: *n*-butanol, acetic acid and water (BAW 4:1:5), phenol and water (PW 3:1) and chloroform, methanol and conc. ammonia (CMA 5:3:1). For HPLC, 0.01 M NH_4OAc was used as starting buffer and then CH_3CN (0-100%). The chromatographic mobility of hypothalamic and pancreatic TRF as measured in radioimmunoassay was in all the systems similar to that of synthetic TRF.

Site	R _F values in TLC			HPLC retention time (min)
	BAW	PW	CMA	
hypothalamus	0.19	0.75	0.88	11
pancreas	0.13	0.75	0.85	12
pancreas plus P	0.15	0.75	0.85	12

Results

TRF immunoreactivity in extracts of rat hypothalamus (Fig. 1 panel b), pituitary brain, spinal cord, pancreas (Fig. 1 panel c), stomach, duodenum and colon was eluted in gel filtration in LH-20 as synthetic TRF (Fig. 1 panel a), before the major part of the proteins and salts. TRF immunoreactive fractions from gel filtration were submitted to SP C 125. The neutral 0.2 M buffer TRF immunoreactivity of the above-mentioned tissues was tested at 0.25-0.75 V₀ at the same volume at which synthetic TRF is eluted. In Fig. 2 the elution of TRF immunoreactivity in hypothalamic and pancreatic samples (panel b and c) is presented and compared with the elution of synthetic TRF (panel a).

Table II Distribution of TRF in various rat tissues. Each sample was extracted and submitted to gel filtration and cation exchange chromatography before radioimmunoassay. Results are expressed as pg of TRF per mg of wet tissue (mean of 4-6 samples \pm S.D.). Recovery of TRF was 90-100%. Note high TRF concentrations in post. pituitary and spinal cord. There are also significant amounts of TRF in pancreas and intestine, but not in liver.

Site	Sample wet weight (mg)	TRF (pg/mg)
total stomach		
hypothalamus	33 \pm 10	410.0 \pm 82.0
anterior pituitary	0.8 \pm 0.1	380.0 \pm 40.0
posterior pituitary	8 \pm 2	18.0 \pm 5.0
testes and epididymis	275 \pm 10	49.0 \pm 15.0
cranial spinal cord	67 \pm 11	49.0 \pm 7.0
thoracic spinal cord	60 \pm 13	84.0 \pm 23.0
lumbar spinal cord	65 \pm 6	115.0 \pm 43.0
crabrovia	1170 \pm 84	2.9 \pm 0.8
intestine	300 \pm 18	1.3 \pm 0.5
intestine and stomach		
intestine	434 \pm 180	1.0 \pm 0.1
intestine	153 \pm 21	0.5 \pm 0.1
intestine	360 \pm 61	0.4 \pm 0.2
intestine	196 \pm 28	0.2 \pm 0.2
intestine	970 \pm 82	0.05

cord, cerebrum, cerebellum, Nervus trigeminus, N. sciatica, pancreas, stomach, duodenum, colon, muscle, lung and kidney. The tissue specimens were weighed and homogenized in 5 parts of 90% methanol (w/v) or 1 ml at least. After centrifugation (10 min at 3000 rpm) the supernatants were evaporated at 40°C under air and the residues were washed with 2 ml of 0.01 M acetic acid and chloroform. The phase was submitted to gel filtration in LH 20 (Pharmacia, Sweden, 1 x 50 cm column) in 100% methanol and 2-4 ml fractions were collected, evaporated, reconstituted in H₂O and measured for conductance, optical density at 260 nm and for TRF in radioimmunoassay. Our anti-TRF is specific to α -glutamate- and Pro-NH₂-moieties, but cross-reacts with His-modifications (Leppälüoto 1975, Ling *et al.* 1976). Luteinizing hormone-releasing factor, somatostatin, thyroglobulin and thyroid hormones do not react. The assay sensitivity is 1 pg.

TRF-containing fractions from gel filtration were submitted to cation exchange chromatography on SP C 25 (Pharmacia) column, (0.8 x 10 cm) equilibrated and washed with 0.01 M ammonium acetate, pH 5.7. TRF was eluted with 0.2 M ammonium acetate, pH 7.4. The fractions were lyophilized as before. The recovery of synthetic TRF (prepared by N. Ling, the Salk Institute, U.S.A.) after LH 20 and SP C 25 was 90-100%.

Partially purified hypothalamic and pancreatic samples were further submitted to thin layer chromatography (Eastman Silica Gel No. 6061) in 3 solvent systems: butanol, acetic acid and water (BAW 4:1:1), phenol and water (PW 3:1) and chloroform, methanol and conc. ammonia (CMA 5:1:1). After runs the sheets were sectioned into 5 mm regions, washed twice with 1 ml of 90% methanol, completely evaporated and measured in TRF radioimmunoassay. Synthetic TRF was mixed with the half of pancreatic sample and used as control. Partially purified hypothalamic and pancreatic samples were also submitted to high-pressure liquid chromatography (Varian, U.S.A., CH-column, 2.1 x 250 mm) with 0.01 M ammonium acetate at pH 4 followed with increasing acetonitrile (0-100%) as described by (Burges *et al.* 1976). Eluates were collected at 1 min intervals, lyophilized and measured in TRF radioimmunoassay.

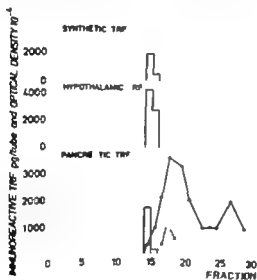


Fig. 1

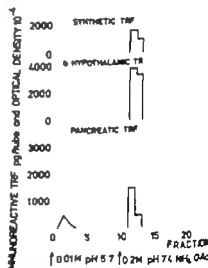


Fig. 2

Fig. 1. Elution profile of immunoreactive TRF extracted from rat hypothalamus (panel b) and pancreas (panel c). Column was LH 20, 1 x 50 cm, eluted with 100% methanol. 4 ml fractions were collected and measured for TRF proteins (optical density, solid line) and conductivity (interrupted line). Elution of synthetic TRF is presented in panel a. Note the similar elution of hypothalamic and pancreatic TRF with synthetic TRF. Other tissues (see Table II) behaved similarly.

Fig. 2. Cation exchange chromatography of purified hypothalamic (panel b) and pancreatic (panel c) immunoreactive fractions from experiment presented in Fig. 1. Column was SP C 25, 0.8 x 10 cm, 4 ml fractions were collected, lyophilized and measured in TRF radioimmunoassay (columns). The elution of synthetic TRF (panel a) is also presented. Hypothalamic and pancreatic TRF were eluted as synthetic TRF. Other tissues (see Table II) behaved similarly.

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In TLCs and HPLC the R_f -values and retention times of TRF immunoreactive purified hypothalamic and pancreatic samples were similar to those in which rat TRF was added (Table I) and only one immunoreactive peak existed.

Quantitatively (Table II) highest TRF immunoreactive concentration was found in hypothalamus, 410 pg per mg of wet tissue, neurohypophysis had 380, spinal cord 8, medulla oblongata 49 and adenohypophysis 18 pg/mg. The extrahypothalamic brain tissue cerebellum had 2.9 and 1.3 pg/mg respectively. The immunoreactive TRF concentration in gastrointestinal tissues varied from 0.2 to 1.0 pg/mg, being highest in the pancreas, in the colon and non-detectable in liver (less than 0.03 pg/mg). Muscle, kidney, lungs and peripheral nerves (N. sciatica and trigeminus) showed non-detectable TRF immunoreactivity (data not shown).

Discussion

In the course of this purification TRF immunoreactivity of hypothalamus, pituitary, spinal cord, pancreas, stomach, duodenum and colon behaved like synthetic TRF. The reason that TRF belongs to the class of peptides, such as substance P, somatostatin, histamine, gastrin and vasoactive intestinal peptide (Pearse 1976), found both in brain and in tissues. The cells producing these peptides may have a common origin in the embryonic neuroectoderm as we suggested before (Leppäluoto *et al.* 1977) and also suggested in a recent report in which TRF was found in the frog skin and retina (Jackson and Reichlin 1977).

Although the TRF concentration in gut tissues is low (0.2–1.0 pg/mg), the total TRF content in rat gut tissues is considerable (10–15 ng). This is comparable with the TRF content found in hypothalamus or in brain. The complex localization of TRF shown in this study lessens the clinical significance of the TRF measurements in plasma or in urine.

The main function of TRF in the Vertebrata is evidently the stimulation of TSH and prolactin release from the pituitary which would explain its high concentration in hypothalamus. The extrahypothalamic distribution of TRF in brain suggests that TRF could operate as a neurotransmitter substance (Jackson and Reichlin 1974; Oliver *et al.* 1974; Hökfelt *et al.* 1975; Kardon *et al.* 1977). This suggestion is further supported by the widespread pharmacological effects of TRF. Many of the functions which TRF affects are mediated via medullary and spinal neurones and thus might well be connected with our finding of a high medullary and spinal cord TRF concentration. It is feasible that TRF would operate as a transmittersubstance in the gastrointestinal tract too. E.g. intravenous administration of synthetic TRF rapidly elicits abdominal sensations in human subjects and increases the intraluminal pressure of rabbit colon (Smith *et al.* 1977). The role of TRF in these new locations remains to be established.

After the submission of this paper we found that Morley and his collaborators have also shown immunoreactivity to occur throughout the rat gastrointestinal tract in *Block m. Digestion Res.* 1977 79 314–318.

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TABLE 1. Urine output (ml/hour) of subjects I-VII as control (C) and ethanol (E) experiment during four periods. N.S. = non-significant.

6-9 p.m.		9-12 p.m.		0-6 a.m.		6-12 m.	
C	E	C	E	C	E	C	E
158	407	158	200	46	28	37	37
537	490	57	153	13	28	30	37
393	527	60	80	20	11	25	27
300	307	83	40	3	31	45	23
150	513	73	143	20	20	20	20
163	457	103	230	20	15	30	31
180	92	103	77	23	57	30	57
227 ± 93		91 ± 33		11 ± 11		40 ± 13	
372 ± 148		159 ± 82		26 ± 15		32 ± 13	
p 0.02		p < 0.05		N.S.		N.S.	

non-dried NaCl. Then, beginning at noon, they were kept in supine position for 22 h and fasted to 6 p.m. At 6 to 9 p.m. they received deionized water 10 ml/kg. Next morning at 8 a.m. they received water 5 ml. At 10 a.m. they assumed standing position, walking slowly around. They received no food until noon. At that they ingested about 20 kcal/kg and 100 mmol sodium and 90 mmol potassium.

Beginning in the morning of the seventh day the same procedure as two days earlier was repeated, except II instead of water in the evening the subjects now received 15% w/v ethanol 10 ml/kg. Blood samples were taken by venipuncture in control and ethanol experiments hourly in the evening from 6 to 10 p.m., at 12 p.m. and at 4, 8 and 10 a.m. and at noon. Urine was collected 6-9 p.m., 9-12 p.m., 0-6 a.m. and 6-12 a.m.

Plasma AVP was determined by radioimmunoassay (Fyhrquist *et al.* 1976). Blood ethanol and serum plasma, potassium and chloride concentrations are measured as described (Yakubri *et al.* 1974). In blood fractions placed in test of Hewlett packard 9810 A calculator was used.

Results

During ethanol experiment urine output significantly exceeded the control value 6-9 p.m. and 9-12 p.m. (Table 1). During the control experiments plasma AVP concentration showed diurnal variation with higher values in the morning at 8 a.m. than in the evening at 6 p.m. ($p < 0.005$). Physiological responses of AVP release (Fyhrquist *et al.* 1976) were seen in a significant decrease of plasma AVP in all subjects after water loading during the period 6-9 p.m. as well as in an increase after getting up in the morning 10-12 a.m. ($p < 0.005$). Water loading suppressed plasma AVP near zero values in 3 subjects (I, II and V) between 6 and 10 p.m., but there were large individual differences in the response of AVP to water. The effect of slight water loading in the morning (8-9 a.m.) was significant during the ethanol experiment ($p < 0.001$), but not during the control one. Higher plasma AVP levels were observed after 6 h of fasting while in a metabolic ward and on controlled sodium and potassium intake than in ambulatory subjects on free water supply (Fyhrquist *et al.* 1976). During ethanol experiment a wide individual variation was seen in plasma AVP concentration. In subjects II, III and IV over 5 times higher AVP concentrations were observed during ethanol intoxication than during the control period. These subjects also had the steepest rise of plasma AVP after getting up at 10 a.m. during hangover and they were the

Plasma vasopressin in ethanol intoxication and hangover

By

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Abstract

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The effect of ethanol intoxication and hangover on immunoreactive plasma arginine vasopressin (AVP) concentration was studied in 7 healthy supine men in controlled clinical conditions. In 6 subjects AVP increased above control values at the time of maximal blood ethanol concentration. The 1st AVP values were observed in the subjects having nausea and vomiting and the worst hangover symptoms. During hangover plasma AVP values were higher than the controls and the response of plasma AVP to upright posture was exaggerated. The dissociation of plasma AVP concentration and ethanol diuresis suggested that the suppression of AVP release is not the sole determinant of ethanol diuresis. The results may indicate that the toxic effects of ethanol and the severity of hangover symptoms are associated with the state of hydration and individual sensitivity of AVP triggering mechanisms.

The diuretic effect of ethanol is thought to be a consequence of suppressed antidiuretic hormone secretion (Wallgren and Barry 1970, Kissin and Begleiter 1971). However clinical and experimental data indicate that besides diuresis, ethanol may cause water retention (Wallgren and Barry 1970, Kissin and Begleiter 1971, Sargent *et al.* 1975). Short-term experiments in humans (Flynn 1959), dogs (Müllerschoen and Riggs 1969) and rats (Muller *et al.* 1975, Linkola 1974) have even shown that after a transient diuretic phase antidiuresis soon follows.

Endocrine events relevant to the antidiuretic phase in ethanol intoxication and hangover have not been thoroughly investigated in man. Thus, there is no data on plasma antidiuretic hormone concentration during the whole period under the influence of ethanol. The present study describes immunoreactive plasma arginine-vasopressin (AVP) concentration during ethanol intoxication and hangover in supine man in controlled clinical conditions.

Materials and methods

7 healthy men, age 25-35, volunteered for the experiments performed under controlled clinical conditions. They received isocaloric diet (about 30 kcal/kg/day) containing 100 mmol sodium and 90 mmol potassium per day for 5 days. In the morning of the fifth day they received 2/3 the calories of that day and all the

subjects (I, II, IV, V and VII) and in ethanol experiment, 8-9 a.m., in all subjects. And fluctuations in plasma AVP during 1 h periods between venipunctures could not be ruled out. This particularly refers to subjects III and VI, in which plasma AVP was not suppressed by water during the first hours of the experiment.

Previous reports (Wallgren and Barry 1970, Kissin and Begleiter 1971, Marquis *et al.* 1975) have emphasized that the diuretic effect of ethanol is caused by AVP suppression. Renal diuresis has been thought to occur as long as blood alcohol concentration rises (Sjgaard *et al.* 1941). In accord with this, we observed initial diuresis and a decrease of plasma AVP during the phase of rising blood ethanol concentration in 5 subjects (II, III, V and VI). Moreover plasma AVP increased above control values at the time of maximal blood ethanol concentration in all but one (VI) subject, and paradoxal AVP peaks were observed in two subjects (III and IV).

In this study higher base-line AVP values were obtained than we have reported elsewhere (Larsson *et al.* 1976). Such high basal AVP levels have not been observed in our later studies, either (unpublished results). Therefore, several possibilities should be considered, explaining high basal AVP levels in subjects III and IV. Fasting for 6 h, controlled sodium-water balance, warm summer during which the experiments were performed and the risk of being on a metabolic ward, *per se* may account for high base-line AVP levels. A possible nonspecific interference of plasma factors cannot be retrospectively excluded. Such factors, however, we have so far encountered in plasma from pregnant women only. In addition in the particular assay in which all samples of subjects III and IV were assayed, high AVP levels may have been also caused by diminished immunoreactivity of AVP standards. However, as it was the aim of this study to compare AVP values after ethanol ingestion relative to control levels, this problem appears a secondary one. In fact, despite several immunoassay methods (Fyhrquist *et al.* 1976, Katz *et al.* 1976, Robertson *et al.* 1973) we yielded plasma AVP levels of roughly the same magnitude in man (0-10 pg/ml), and are aware of no study giving the absolute values of AVP in human plasma at various levels of hydration and in controlled clinical conditions.

In spite of higher AVP concentrations, initial ethanol diuresis exceeded control values in subjects (II and III). During the period 8-12 a.m. there were no significant difference in urine output between ethanol and control experiment, in spite of higher plasma AVP concentrations in ethanol experiment. Although specific evidence is lacking, some explanations for this apparent discrepancy also observed in subjects I, V and VII, and in mean values, may be offered. Thus, catecholamines, the synthesis and release of which is stimulated during ethanol intoxication (Wallgren and Barry 1970, Kissin and Begleiter 1971), may affect urine output. Noradrenaline has been described to counteract the cellular actions of vasopressin at the level of cyclic AMP synthesis (Klein *et al.* 1971, Beck *et al.* 1972), and dopamine has been reported to increase renal blood flow, glomerular filtration rate and sodium excretion (McDonald *et al.* 1964). Ethanol might also delay the event between AVP receptor attachment and increase of luminal membrane water permeability in the distal collecting ducts, thereby causing a time lag between peaking plasma AVP values and antidiuresis. It is unlikely that ethanol would reverse AVP induced antidiuresis in man, because the long-acting AVP analogue, 1-desamino-D-arginine vasopressin (Ferring, Malmö,

TABLE II Serum sodium (Na), potassium (K) and chloride (Cl) concentration (mmol/l) before and after ethanol (E) ingestion (6 p.m.) and next morning (8 a.m.) and the corresponding mean \pm S.D. Values of subject I are falling.

	[Na ⁺]				[K ⁺]				[Cl ⁻]			
	6 p.m.		8 a.m.		6 p.m.		8 a.m.		6 p.m.		8 a.m.	
	C	E	C	E	C	E	C	E	C	E	C	E
II	140	141	140	144	3.9	4.0	4.3	4.3	106	106	105	105
III	140	140	141	145	4.0	3.8	4.7	4.7	104	104	102	102
IV	140	143	138	145	4.2	3.8	4.5	4.3	104	104	104	104
V	141	142	141	145	4.2	4.0	4.4	4.2	103	103	104	104
VI	141	141	140	143	3.6	3.9	4.3	4.4	105	105	106	106
VII	141	142	140	145	4.1	4.3	4.2	4.5	105	104	106	106
	141	142	140	^a 145	4.0	4.0	^b 4.4	4.4	105	104	105	105
	± 0.6	± 1.1	± 1.1	± 0.8	± 0.2	± 0.2	± 0.2	± 0.2	± 1.1	± 1.0	± 1.5	
	$p < 0.05$		$p < 0.001$									

differs not significantly from the value at 6 p.m.

^a $p < 0.05$ with respect to the value at 6 p.m.

^b $p < 0.005$ with respect to the value at 6 p.m.

^c $p < 0.001$ with respect to the value at 6 p.m.

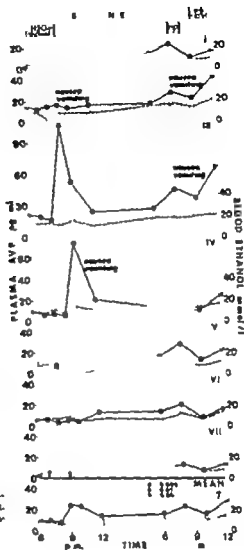
only subjects to have nausea and vomiting during the experiments. They also were the ones experiencing subjective hangover symptoms (Ylikahri *et al.* 1974). Nausea and vomiting were temporally related to the AVP peak in subject IV but not in subject III. A clear AVP peak was observed in the case exhibiting the most serious signs of emesis (subject II).

When ethanol and control experiments were compared subjects II and III had a discrepancy between plasma AVP and the diuretic response 6–12 p.m. Thus, antidiuresis did occur despite increased AVP levels. Similar discrepancy was observed in subject I, 4–6 p.m., in subject V 9–12 p.m. and 0–12 a.m. and in subject VII 9–12 p.m. and 0–12 a.m. After midnight plasma AVP values were significantly higher in the ethanol experiment except after water ingestion in the morning. However no significant difference was observed in urine output between ethanol and control experiment between 0 and 12 a.m. The rise in plasma AVP was steeper during hangover 10–12 a.m. than during the corresponding control time.

Serum sodium and chloride concentration rose during ethanol experiment but not during control experiment (Table II). Serum potassium concentration increased in both control and ethanol experiment.

Discussion

Diurnal variation in plasma AVP was observed during the control experiment. After overnight water deprivation, at 8 a.m., there were higher plasma AVP values than in the evening at 6 p.m. in accord with the data of Katz *et al.* (1976). Possible secretory spurts as reported by Katz *et al.* (1976) could not be distinguished because of the long time intervals between collecting blood samples. Water ingestion between 8 and 9 p.m. suppressed plasma AVP



1. Plasma vasopressin (AVP) of subjects I-VII at control (O) and ethanol (●) experiments. Dashed line represents blood ethanol concentration (not measured in I).

Thus, also alternative explanations should be offered for increased AVP response to alcohol. It has been concluded that potassium depletion stimulates thirst (Berl *et al.* 1977). During water experiment subjects II, III and IV had the highest urine output between 6 and 12 p.m. (Table I). This indicates high water intake while on the controlled diet. These factors and low initial serum [K⁺] in subjects III and IV before ethanol ingestion (Table II) may indicate hypokalemia in these subjects (Berl *et al.* 1977). On the other hand subject VII had high serum [K⁺] at 6 p.m. Sodium ions have been suggested to have a specific role in AVP release and thirst (Anderson 1977). Present results may provide a parallel explanation. Thus, potassium deficiency may as well have sensitized AVP releasing mechanisms. It is tempting to hypothesize that the mechanism is based on the function of Na⁺-K⁺ ATPase,

Sweden) abolished ethanol diuresis (M. M. Nieminen *et al.*, to be published). In a experiment at 6 p.m. subjects III and IV had the lowest serum $[K^+]$ (Table II). In a previous study (Linkola *et al.* 1976) decrease of serum $[K^+]$ was observed during ethanol intoxication. In potassium depletion the renal response to vasopressin is blunted (Palsson 1970). This may partly explain co-existing high urine output and high AVP values.

Dehydration following higher urinary output after ethanol than after water loading (Table I) may physiologically explain high plasma AVP levels after ethanol loading. Serum $[Na^+]$ (Wallgren and Barry 1970, Kissin and Begleiter 1971, Robertson *et al.* 1976) and activated renin-angiotensin system (Linkola *et al.* 1976, Keil *et al.* 1973, Fama and Fabre 1975) may stimulate AVP release during ethanol intoxication. In animals, simultaneous administration of hypertonic sodium chloride solution and angiotensin II has a synergistic effect in stimulating AVP release (Andersson 1971). In subject III the rise in serum $[Na^+]$ and plasma renin activity (reported elsewhere) were the most pronounced during ethanol experiment. This may indicate the role of renin-angiotensin system and sodium ions in AVP release in subject III. The reduction of blood volume following diuresis may also lower the threshold of osmoreceptors (Dunn *et al.* 1973). Blood pressure was not monitored in this study and nausea may also have affected the results.

Among the most important factors regulating vasopressin release is serum osmolality (Robertson *et al.* 1976, Hays 1976) which parallels blood ethanol concentration (Räbergh *et al.* 1972, Robinson and Loeb 1971, Champion *et al.* 1975). Osmoreceptors, though sensitive to hypertonic solutions (Hays 1976), could be stimulated by ethanol only if it reacts to raised tonicity which means the change of colligative properties of blood, not by ethanol. As ethanol diffusing freely into cells, it is incapable of increasing effective osmotic pressure (Wallgren and Barry 1970), ethanol could hardly stimulate AVP release by directly increasing the osmotic pressure at osmoreceptive cells. Present knowledge of osmoreceptors is insufficient to permit further evaluation of this problem. It should be, however, noted that ethanol doses used in this experiment may increase serum osmolality also (Linkola *et al.* 1976). The intra- and extracellular effects of this increment, *per se*, are not known but even the sympathetic stimulation after ethanol has been thought to be due to the osmotic effects of ethanol (Regan *et al.* 1966). Effects of ethanol on autonomic neurons surrounding supraoptic nuclei cannot be excluded as a cause of AVP release, as supporting data on this area is lacking.

High plasma AVP levels during hangover were probably due to dehydration, as indicated by increased serum $[Na^+]$ levels (Table II), and possibly to activated renin-angiotensin system (Linkola *et al.* 1976). During hangover the rise of plasma AVP in response to upright posture was exaggerated (Fig. 1). Lowered plasma volume due to dehydration may have increased the postural stimulus of upright position (Brown *et al.* 1966).

Higher plasma AVP values in subjects II and III before ethanol ingestion (Fig. 1) reflect their lower degree of hydration as related to the control experiment. Such a degree of hydration may potentiate the effect of ethanol on plasma AVP because in subjects I and VII having lower starting levels of AVP in ethanol experiment than in control experiment the response of plasma AVP to ethanol was weak. However initial serum $[Na^+]$ in subjects II and III did not indicate dehydration when related to the control value.

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but this remains to be confirmed. Whether stimulating effects of ethanol on this were related to AVP release remains also to be studied. Potassium deficiency may have been partly responsible for high basal AVP levels and poor AVP response in some subjects. Prostaglandins may also have contributed to high AVP values (Aske 1977), as well as to co-existing diuresis and natriuresis in subject III, 6-12 p.m., in experiment, but supporting data is lacking.

While AVP release appears to be associated with nausea (Shelton *et al.* 1977) this hardly explain high AVP in subject III (Fig. 1). Because also in subject II emesis was correlated to plasma AVP it is possible that at least during ethanol intoxication and over there is no absolute association between nausea and vomiting and AVP release. It is also supported by the findings of Hussain *et al.* (1975). Decreased blood pressure, a powerful stimulus for AVP release (Robertson *et al.* 1973), may occur during heavy ethanol intoxication (Wallgren and Barry 1970) but was not studied in this work. On the other hand the moderate ethanol dose given did probably not cause blood pressure reduction sufficient to explain AVP release in our study.

Pronounced AVP increments after ethanol may thus reflect individual sensitivity of the hypothalamic AVP releasing mechanisms. Subjects II, III and IV, having the largest difference in plasma AVP between ethanol and control experiments, also were the most severely intoxicated ones (nausea and vomiting, Fig. 1) and had the worst subjective hangover symptoms. These associations, although difficult to assess on the time axis, may be common denominators of decreased alcohol tolerance, as indicated by emesis and hangover symptoms. To explain raised plasma AVP levels in ethanol intoxication and hangover, we favor the principal mechanisms as previously discussed: 1. dehydration and the increased blood [Na⁺] due to ethanol diuresis, 2. stimulated renin-angiotensin system, 3. mechanism associated with emesis or blood pressure decrease in some subjects and 4. raised osmolality of body fluids, 5. potassium depletion, prostaglandins or other factors, which were not controlled here.

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ing the hormone releasing mechanisms in the pancreas. Our interest in such a possible control of pancreatic hormone release by vascular receptors originates from previous studies on the plasma volume control during bleeding (Järhult *et al.* 1972, Järhult 1973 *et al.* & Grönroos 1975, Järhult 1975 a, Järhult *et al.* 1976). These studies clearly indicated an important contribution to the plasma volume restoration after bleeding is made by osmotic absorption of extravascular fluid into the blood stream, in turn due to a decrease in the plasma glucose concentration. The hemorrhagic hyperglycemia was found to be caused by a release of glucose from the liver and the effect seemed to be mediated via several different links of the sympatho-adrenal system (Järhult 1975 b). With regard to pancreatic hormone release during hemorrhage, several studies have demonstrated that bleeding is associated with an increased plasma level of glucagon (*e.g.* Halmagyi *et al.* 1973, Järhult 1975 b, Lindsay Faloona & Unger 1975) and, in spite of the hyperglycemia, unchanged or even lowered plasma insulin concentration (*e.g.* Carey Lowrey & Clouder Moss *et al.* 1970, Hiebert *et al.* 1973).

In a recent publication we presented experimental data which indicated that the reflex glucagon release in hemorrhage is elicited preferentially from arterial baroreceptors, whereas aortic chemoreceptors and cardiopulmonary receptors play only minor roles (Järhult, Öberg & Lundvall 1977). It therefore seemed reasonable to suppose that arterial baroreceptors also may by reflex influence the secretory pattern of the islets of Langerhans, and present investigation was undertaken to test this hypothesis. Emphasis was placed on analysis of the efferent pathways in such a reflex, since in recent years it has been shown that the endocrine pancreas can be strongly influenced not only by adrenal catecholamines, but also by direct sympathetic nerve fibres (Esterhuizen & Howell 1970, Marliss *et al.* 1973, Bloom, Edwards & Vaughan 1973, Porte *et al.* 1973, Bloom & Edwards 1975). Preliminary data from this work has been published elsewhere (Järhult & Holst 1977 *et al.*, Lagermanne & Holst 1977).

Methods

The study was performed on 22 cats of both sexes (mean weight 3.8 kg). The animals were fed on normal chow diet, but fasted 10–16 h before the start of the experiment. Anaesthesia was induced with ether and maintained with a mixture of chloralose (30 mg/kg) and urethane (30 mg/kg) given i.v. A tracheal cannula was inserted and the animals breathed spontaneously throughout the experiment.

Interference with afferent carotid baroreceptor activity. In all experiments interference with the normal afferent input from the carotid baroreceptors was made 45 min after bilateral vagotomy in the neck. In 17 experiments complete denervation of this afferent inhibitory influence was accomplished by bilateral section of the glossopharyngeal nerve, which was dissected free 6–8 mm from the carotid bifurcation. In 4 other experiments, the carotid baroreceptor activity was changed by varying the distending blood pressure in isolated carotid arteries. For this purpose, the right iliac artery was cannulated with polyethylene tubing and the arterial outflow diverted to the two common carotid arteries via Harvard peristaltic pumps. The external carotid arteries were cannulated with polyethylene tubing about 1 cm distal to the carotid bifurcation and the outflows diverted to T-tubes inserted into the right jugular vein. All other carotid vessels and on the right iliac artery. The intraluminal pressure was recorded on each side with a P 23 AC transducer connected to a T-tube in the aorta close to the carotid bifurcation. With this technique, the mean distending pressure in the carotid sinus could be changed to the desired levels by adjustment of the pump and the screw clamps.

Interference with the efferent sympatho-adrenal system. In order to study the efferent adrenergic path-

Reflex adrenergic control of endocrine pancreas evoked by unloading of carotid baroreceptors in cats

By

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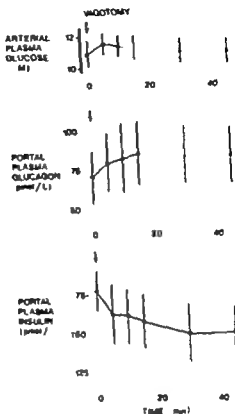
Abstract

JÄRHULT J and J J HOLST *Reflex adrenergic control of endocrine pancreas evoked by unloading of carotid baroreceptors in cats* Acta physiol. scand 1978 104 188-202

The effects of unloading of the carotid baroreceptors on arterial plasma glucose concentration and on portal plasma immunoreactive glucagon (IRG) and insulin (IRI) concentrations were studied in anesthetized, vagotomized cats either by sectioning the sinus nerves or by lowering the pressure in the carotid sinuses. Complete elimination of the carotid baroreceptor discharge by cutting the sinus nerves caused an increase in the arterial plasma glucose concentration by 100% and an increase in the portal IRI level by about 200% whereas the portal IRG concentration decreased to 50% of its basal value. Baroreceptor-induced changes of the plasma IRG and IRI levels seemed to be graded in relation to drop in carotid blood pressure and they were clearly detectable when the pressure was lowered to 90 mmHg in the isolated carotid sinus preparation. The described reflex hyperglycaemia, hyperinsulinaemia and hypoinsulinaemia were mediated to the pancreas and liver mainly by the sympathetic system, since cutting the splanchnic nerves above the adrenal glands abolished the hyperglycaemic, hypoinsulinaemic responses and markedly depressed the magnitude of the hyperglucagonaemic response. In adrenalectomized cats, complete unloading of the baroreceptors evoked both hyperglucagonaemia and hypoinsulinaemia although the magnitude of the hormonal responses was diminished. In animals with the pancreas and liver were sympathetomized but the adrenal glands left intact, cutting the sinus nerves evoked a doubling of the IRG level and a slight increase in plasma glucose, but no significant change in the IRI level. I.v. infusion of adrenaline (1 µg/kg min) or noradrenaline (5 µg/kg min) caused pronounced increases in IRG and plasma glucose and a near-total reduction of IRI. We conclude that function of the endocrine pancreas in the cat can be influenced by variations in the blood pressure by way of a reflex control which originates from arterial baroreceptors. This reflex adjustment of the endocrine pancreas is mediated chiefly by two links of the sympatho-adrenal system, namely by catecholamine release from the adrenal medulla and, more importantly, by a direct adrenergic nerve fibre influence on the α and β -cells.

Key words: Baroreceptors, insulin, glucagon, glucose, sympatho-adrenal system

The literature on the physiology of baroreceptors is extensive (for reviews see Arias & Schmidt 1955; Heymans & Neil 1958; Smith 1974; Kirchheim 1976). Yet there seems to be no report so far on a possible influence from the baroreceptors on metabolic



1. Effects of bilateral cervical vagotomy on arterial plasma glucose concentration and on portal plasma immunoreactive glucagon and insulin concentrations. Data are given as mean values \pm from 11 expts.

arterial blood pressure (AP), heart rate (HR), arterial plasma glucose concentration and portal plasma immunoreactive glucagon (IRG) and insulin (IRI) concentrations were followed during 45 min. As can be seen from Fig. 1 vagotomy caused small but significant increases of IRG and IRI, whereas plasma glucose remained virtually unchanged. The IRG is increased from a control value of 70 ± 17 pmol/l to 85 ± 19 pmol/l 45 min after vagotomy ($p < 0.02$) and the corresponding IRI values were 177 ± 10 and 150 ± 18 pmol/l ($p < 0.02$), respectively. The cardiovascular effects of cervical vagotomy consisted of a moderate, significant increase in HR and an insignificant decrease in AP.

Cardiovascular effects of complete elimination of the carotid baroreceptor discharge

Stimulating the vagus nerves evoked an immediate baroreceptor reflex consisting of tachycardia and increased blood pressure (Fig. 2). The heart rate rose immediately by about 50 beats/min and this level was maintained during the 45 min period of observation. AP showed a small peak response and then gradually returned towards the control level. These reflex cardiovascular responses were seen in all animals, but the magnitude of the reflex responses varied somewhat between the 4 different groups of animals. The increase in AP was thus most prominent in cats with pancreatic sympathectomy or in cats with sectioned splanchnic

ways in the baroreceptor induced adjustments of endocrine pancreas, the changes of glucose, and insulin concentrations in response to sinus nerve sectioning were followed after exclusion of all links of the sympatho-adrenal system. In 5 cats the adrenal glands were removed bilaterally and it was taken to leave the direct sympathetic fibres to other regions intact ("Adrenalectomized" cats). In 5 cats the sympathetic nerve fibres below the adrenal glands were removed by cutting all nerves at the celiac, mesenteric and hepatic arteries as well as in the mesenterium. In these expts. great care was taken to avoid damaging the preganglionic sympathetic nerves to the adrenal glands ("Cats with sympathectomy"). In 4 cats the major and minor splanchnic nerves were cut bilaterally and the rest removed ("Cats with sectioned splanchnic nerves"). 4 cats with an intact sympatho-adrenal system served as controls ("Intact cats").

Cardiovascular recordings and blood sampling. After heparinization (1 000 IU/kg b.w.t.), the right carotid artery was cannulated with a polyethylene tubing for pressure recording and sampling of arterial blood for plasma glucose determination. The arterial blood pressure (AP) was recorded with a Statham P23 transducer and heart rate (HR) was registered with a tachograph triggered by the systolic pressure. A small jejunal vein was cannulated and a polyethylene catheter advanced until its tip was near the portal vein. From this catheter blood samples were withdrawn for analysis of portal plasma concentrations of glucagon (IRG) and insulin (IRI) concentrations. AP and HR were recorded on a Goniograph.

Hemorrhage. In order to investigate if hypovolemia could further augment the baroreceptor-induced hyperglycemia, hyperglucagonemia and hypoinsulinemia, hemorrhage was performed in 4 cats with intact sympatho-adrenal system 45 min after sectioning the sinus nerves. The hemorrhagic hypotension was accomplished by bleeding the animal into a silicized glass bottle connected via a T tube to the cannulated right brachial artery. The bleeding was rapid so that the arterial pressure reached 30 mmHg within 5 min and this level was then maintained during 30 min with the aid of the pressure bottle.

Drug infusions. In order to compare the effects on endocrine pancreas of endogenously released catecholamines with those obtained by exogenous administration, adrenaline or noradrenaline was infused 45 min after cutting the sinus nerves. The substances were dissolved in saline and infused at a rate of 0.5 ml/min in a dose of 1 or 5 µg/kg b.w.t. × min for 10 min. Blood samples were drawn at 10, 20 and 35 min after the end of infusion.

Protocol. After completion of the surgery the animals rested for 30 min until then the vagus nerves were cut bilaterally in the neck. In 11 cats blood samples were drawn before vagotomy and then 3, 10, 20 and 45 min after vagal denervation, whereas 10 cats rested 45 min after the vagotomy before blood sampling. The sinus nerves were then cut bilaterally in 17 cats and blood samples for glucose, glucagon and insulin analyses drawn before and 3, 6, 10, 15, 20, 25 and 45 min after the sinus nerve denervation. In 4 cats prepared with isolated carotid sinuses, the mean distending blood pressure of the carotid sinuses were lowered from the normal value (≈ 120 mmHg) to about 90 mmHg and then to about 60 mmHg. Each hypotensive period lasted for 10 min and blood samples were drawn at 3, 6 and 10 min.

After this observation period, hemorrhage to 30 mmHg was performed in 4 expts., adrenaline 10 µg/kg for 10 min in 4 expts. and noradrenaline infusion for 10 min in 8 expts. Blood samples from the brachial and portal veins were drawn 3, 6, 10, 15, 20 and 45 min after the start of catecholamine infusion and 10, 20 and 30 min after the start of hemorrhage.

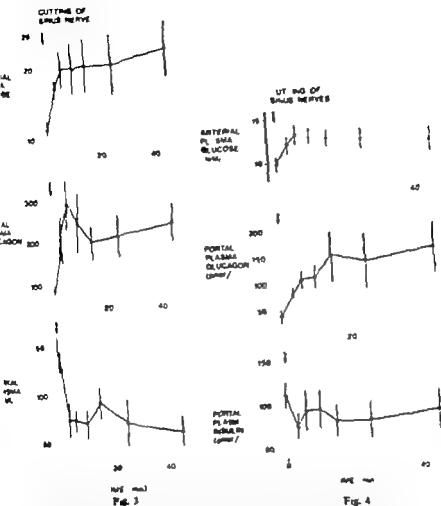
Biochemical analyses. Arterial plasma glucose concentration was determined on the autoanalyser with a glucose-oxidase system (Trinder 1969). Portal blood was collected in ice-chilled tubes containing heparin (50 U) and aprotinin (Trasyol, Bayer GRF) 500 kIU/ml blood. Portal plasma insulin was determined according to Albano *et al.* (1972) and portal plasma glucagon concentrations by radioimmunoassay as previously described (Holst, Christiansen & Köhl 1976). The antiserum used is directed against the terminal sequence of glucagon and crossreacts less than 0.1 per cent with high concentrations of insulin, and the inter-assay coefficient is 15 per cent.

Statistical methods. All data are presented as mean values ± S.E. The *t*-test for paired observations was employed for statistical evaluation.

Results

Effects of cervical vagotomy

Bilateral vagotomy in the neck was made in all expts. In order to facilitate the observation of the baroreceptor response. In 11 of these expts. the effects of cervical vagotomy *per se* on systemic



3 Effects of bilateral cutting of the splanchnic nerves on arterial plasma glucose concentration and on portal plasma adrenomedullary glucagon and insulin concentrations in vagotomized cats with an intact splanchnic-adrenal system. Data given as mean values \pm S.E. (n = 4).

4 Effects of bilateral cutting of the splanchnic nerves on arterial plasma glucose concentration and on portal plasma adrenomedullary glucagon and insulin concentrations in vagotomized, adrenalectomized cats. Data are expressed as mean values \pm S.E. (n = 3).

rates and a similar quantitative difference was noted also with regard to the changes of heart rate.

Effects on glucose, glucagon and insulin of complete ablation of the splanchnic baroreceptor discharge

Experiment 1. Cutting the splanchnic nerves caused a characteristic response pattern consisting of hypoglycemia, hyperglucagonemia and hypoinsulinemia (Fig. 3). The arterial plasma glucose concentration rose from control level of 11.4 ± 1.6 mM to a maximum level of 22.5 ± 4.1

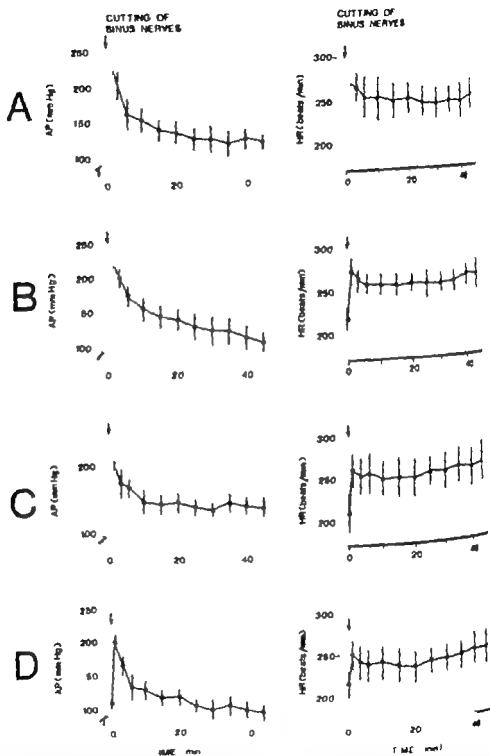


Fig. 2. Effects of bilateral cutting of the sinus nerves in vagotomized cats on arterial blood pressure and heart rate (H.R.). Data are given as mean values \pm S.E. Panel A shows the responses in intact cats ($n=4$), panel B the responses in adrenalectomized cats ($n=5$), panel C the responses in cats with partial sympathectomy ($n=4$), and panel D the responses in cats with sectioned splanchnic nerves ($n=4$).

1 Effect on mean systemic arterial blood pressure (AP), heart rate (HR), arterial plasma glucose concentration, and on portal plasma glucagon and insulin concentrations evoked by stepwise reduction of the isolated carotid sinus pressure from about 120 mmHg down to 90 and 60 mmHg, respectively. The animals were vagotomized in the neck about 45 min before experimental intervention. Data are expressed as mean values \pm S.E. from 4 experiments.

	Carotid sinus pressure ≈ 120 mmHg	Carotid sinus pressure ≈ 90 mmHg			Carotid sinus pressure ≈ 60 mmHg		
		3 min	6 min	10 min	3 min	6 min	10 min
mmHg	126 ± 6	192 ± 16	171 ± 19	163 ± 19	183 ± 19	156 ± 13	130 ± 18
(beats/min)	207 ± 11	231 ± 8	229 ± 9	229 ± 8	243 ± 13	243 ± 12	243 ± 10
glucose (mM)	11.8 ± 1.3	13.1 ± 1.5	14.2 ± 1.6	14.4 ± 1.5	15.9 ± 1.5	17.1 ± 1.7	17.5 ± 1.6
glucagon (pmol/l)	176 ± 14	284 ± 44	331 ± 95	335 ± 90	366 ± 115	418 ± 97	449 ± 136
insulin (pmol/l)	77 ± 23	51 ± 14	51 ± 15	49 ± 14	41 ± 12	47 ± 18	48 ± 21

to a similar degree in percent after sinus nerve sectioning. The IRI concentration raised 15–30% from control which is to be compared with the 50% decrease observed in intact cats.

after the pancreatic sympathectomy As can be seen from Fig. 5, cutting the sinus nerves in these animals caused an increase in not only the concentration of IRG and arterial plasma glucose but also of IRI. The glucose concentration rose by only 3 mM as a maximum, but concentration of IRG rose to a level twice that of the control values or from 68 ± 25 pM to about 140 pM/l. The increase in IRI was slight and not statistically significant.

after the sectioned splanchnic nerves Fig. 6 illustrates that sectioning of the sinus nerves in these animals did not significantly change the plasma glucose or IRI concentration. Plasma glucose tended to decrease gradually during the observation period and IRI to rise, i.e. the changes were opposite to those in the intact animals. However there occurred a moderate increase in IRG from 60 ± 13 pM/l to about 130 pM/l in response to the complete baroreceptor unloading.

Effects on glucose, glucagon and insulin of stepwise reduction of carotid pressure

The mean distending pressure of the isolated carotid sinus regions was reduced in steps to a normal AP of about 120 mmHg down to 90 and 60 mmHg, respectively. It was found that there existed a close correlation between the mean distending pressure and the plasma levels of glucose, glucagon and insulin (Table 1). Clear-cut changes in hormone concentrations were observed even if the mean carotid pressure was lowered only to 90 mmHg, when the glucagon level was increased by about 100% and the insulin level depressed by about 50%.

Effects on glucose, glucagon and insulin of hemorrhagic hypotension

In 4 cats with an intact sympatho-adrenal system, hemorrhagic hypotension to 50 mmHg was made 90 min after cervical vagotomy and 45 min after bilateral sinus nerve section. The effects on glucose, glucagon and insulin levels of this hypotension are depicted in Fig. 7. As can be seen, there was a dramatic increase both in the arterial plasma glucose concentra-

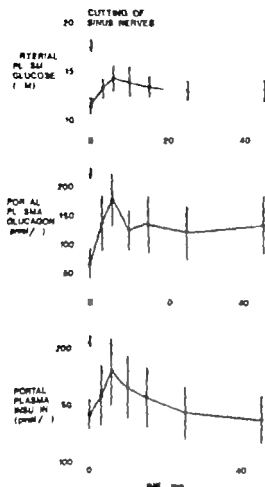


Fig. 5

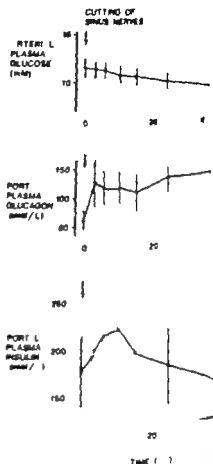


Fig. 6

Fig. 5. Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and portal plasma immuno-reactive glucagon and insulin concentrations in vagotomized cats. All portal sympathetomy. Data are given as mean values \pm S.E. ($n=4$).

Fig. 6. Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and portal plasma immuno-reactive glucagon and insulin concentrations in vagotomized cats. All splanchnic nerves. Data are given as mean values \pm S.E. ($n=4$).

mM after 45 min, but a clear-cut increase was observed as early as 3 min after sectioning the sinus nerves. IRI rose from a control value of 88 ± 5 pmol/l to a peak value of 261 pmol/l after 6 min and it then remained virtually constant at a level 2–3 times above control. The control value of IRI was 143 ± 19 pmol/l but IRI very quickly dropped to about 9% of its control value and the hypoinsulinemia was maintained during 45 min of observation.

Adrenalectomized cats Fig. 4 illustrates that the adrenalectomized cats showed the same pattern of hormonal response to sinus nerve sectioning as the intact ones, though with quantitative differences. Thus, the average glucose concentration increased only about 1 mM in these cats; one cat, in fact, did not change its plasma glucose level at all. The IRI concentration in the control period was lower in these cats than in the intact ones, but

pancreas. As can be seen from Fig. 8, there was a considerable difference in the glucose and insulin responses to infusion of A and NA in a dose of $1 \mu\text{g}/\text{min} \times \text{kg b.wt.}$ Increases in glucose and IRG were thus much more pronounced for A than for NA. With regard to IRI, infusion of A caused a clear-cut decrease of IRI, whereas NA, if blug, tended to increase the IRI level. However an infusion of NA in a dose of $5 \mu\text{g}/\text{kg b.wt.}$ evoked a clear-cut increase in arterial plasma glucose concentration and a plasma glucagon concentration and a moderate decrease in portal plasma insulin concentration, i.e. changes very similar to those elicited by A. — The cardiovascular effects of the two substances were very much the same.

Discussion

Recent work in our laboratory has demonstrated that a significant part of the plasma glucose restoration after bleeding is accomplished by a glucose-osmotic absorption of extracellular fluid into the circulation, in turn due to an increased output of glucose from the liver (see Introduction). In a recent publication we have presented experimental data suggesting that this hepatic glucose release is caused preferentially by a reflex initiated from arterial baroreceptors (Järnåhlt, Holmberg & Lundvall 1977). Since hemorrhage is associated with increased plasma glucagon concentrations (Halmagyi *et al.* 1969 Järnåhlt 1975 b, deasy Faloon & Unger 1975) and suppressed insulin release (Carey Lowrey & Cloutier 1970, Moss *et al.* 1970, Häbert *et al.* 1973), we found it worthwhile to investigate if the feline pancreas also could be reflexly controlled from the arterial baroreceptors and, further if such a reflex could account for the observed hormone adjustments in bleeding. We have found that complete elimination of the afferent baroreceptor discharge by slow section evoked a distinct pattern of response in terms of hyperglycemia, hyperglucagonemia and hypoinsulinemia. Complete elimination of the baroreceptor discharge is equivalent to a drop in arterial blood pressure to about 50–60 mmHg but the experiments with isolated cold sinuses showed that a clear-cut increase in glucagon and decrease in insulin concentration occurred also when the arterial blood pressure fell from 120 down to 90 mmHg. Thus, the adjustments of endocrine pancreas from carotid baroreceptors seem to be operating in a wide physiological range of arterial blood pressure. The described response pattern is present also after adrenalectomy whereas cutting the pancreatic sympathetic nerves abolished the inhibiting effect on insulin secretion. Bilateral section of the splanchnic nerves abolished both the hyperglycemia and hypoinsulinemia and diminished the hyperglucagonemic response. Exogenous catecholamines had effects similar to those observed during sinus denervation in the intact cat. These findings indicate that the reflex is mediated by the islets chiefly by the sympatho-adrenal system and that the adrenal catecholamines as well as activation of direct sympathetic nerves contribute to the full effect. The fact that a certain increase of glucagon was present also after splanchnic nerve section might be taken to indicate that the hyperglucagonemic response in part is due to a prolonged elimination time (see Lefebvre & Luyckx 1976). A decrease of the portal insulin concentration was observed after baroreceptor-unloading in the intact and in the adrenalectomized cat, but not in cats with pancreatic sympathectomy. These data strongly suggest that the direct sympathetic innervation conveys an important

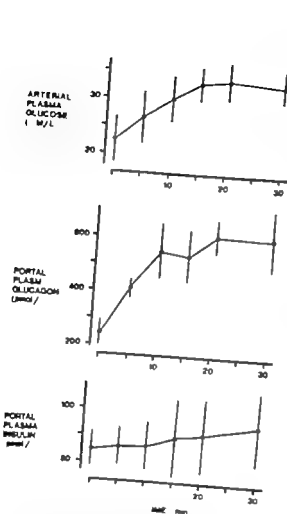


Fig. 7

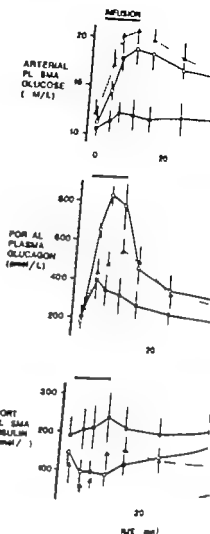


Fig. 8

Fig. 7 Changes of arterial plasma glucose concentration and of portal plasma immuno-reactive glucagon and insulin concentrations in response to hemorrhagic hypotension to 50 mmHg. The hemorrhage was performed 45 min after bilateral cutting of the sinus nerves in 4 cats with an intact sympathetic system. Data are given as mean values \pm S.E.

Fig. 8 Effects of LV infusion of adrenaline (O—O) or noradrenaline (●—●) in a dose of 5 μ g/min kg b.wt. or of noradrenaline (A—A) in a dose of 1 μ g/min kg b.wt. on arterial plasma glucose concentration and on portal plasma immuno-reactive glucagon and insulin concentrations. Data are given as mean values \pm S.E., $n=4$ in each group.

tion and in the portal plasma IRG concentration in response to the bleeding, whereas portal IRI level was very little affected. As a maximum, plasma glucose increased from 22.5 ± 4.1 to 33.8 ± 3.2 mM IRG from 245 ± 45 to 627 ± 128 pmol/L, and IRI from 62 to 85 ± 35 pmol/L. It should be noted that the control values at time zero in Fig. 7 are identical with the values obtained after 45 min in Fig. 3.

Effects on glucose, glucagon and insulin of exogenous catecholamines

Adrenaline (A) or noradrenaline (NA) were infused LV in 12 cats in order to get a comparison with the hormonal response evoked by reflex adrenergic activation of the cat

the observed changes of portal insulin and glucagon levels most likely can be ascribed to altered rates of hormone release.

The present data on the glucose, glucagon and insulin concentrations taken together indicate that there is no distinct relation between the blood sugar level on the one hand and insulin, glucagon, or the insulin/glucagon ratio on the other. This is not unexpected in view of the recent finding that the glucose-releasing effect of glucagon on the liver is not altered until the insulin concentration is depressed. Thus, at normal insulin levels, even physiological doses of glucagon given in the periphery or in the portal vein do not alter the blood glucose concentration (Sherwin *et al.* 1976, Felig *et al.* 1976, Hobt *et al.* 1977). These observations are corroborated by our results. Hence, a pronounced glucose response was noted in the intact and also in the adrenalectomized cats (Fig. 3 and 4), in which the elevated glucagon was combined with a depressed insulin level. On the other hand, in cats with cut splanchnic nerves (Fig. 6) a doubling of the portal glucagon level was ineffective in raising the glucose level in face of a maintained portal insulin concentration.

It should also be noted in this context that the release of glucose from the liver is not only affected by pancreatic hormones but is also under effective control of hepatic sympathetic nerves and of humoral catecholamines from the adrenal medulla (Shimazu & Amakawa 1971, Edwards 1971, 1972 a, b, Edwards & Silver 1972, Järhult 1975 b). It should also be borne in mind that the glucose response is quantitatively related to the amount of glycogen stored in the liver at the beginning of the experiment. Thus, animals with small glycogen stores can develop much less of a hyperglycemic response to splanchnic nerve stimulation than animals with filled glycogen stores (Bloom & Edwards 1975). No glycogen determinations were made in the present series of experiments, but it seems likely that the glycogen stores were the same in all cats since they were all in the immediate postabsorptive state.

The problem whether the sympatho-adrenal system participates in the control of insulin and glucagon liberation during basal conditions is so far not entirely settled. Some authors have reported that α -adrenergic blockers increase and β -adrenergic blockers decrease the plasma insulin level in fasting untrained man (Porte 1967) and in the unanesthetized mouse (Andriquet 1972 a, b), but these findings in man could not be confirmed by Base *et al.* (1970) or by Imura *et al.* (1971). Furthermore, the insulin and glucagon levels at rest are normal both in sympathectomized and adrenalectomized man (Brodows, Pi-Sunyer & Campbell 1974, Järhult, Ingemansson & Hobt to be published). The results reported herein show that the control insulin concentration was higher and the control glucagon concentration lower in cats with cut splanchnic nerves than in those with intact nerves, which might be taken to support a role for the sympatho-adrenal system in basal pancreatic hormone secretion.

The cardiovascular function of the carotid baroreceptors is to maintain the systemic blood pressure at a normal level by continuous adjustments of the heart and peripheral blood vessels. Since the brain is almost completely dependent on blood glucose for its energy requirements, it seems reasonable that the carotid receptors also should be able to control the release of glucose into the circulation, so that they in situations of cerebral hypotension or hypoperfusion (such as in hemorrhage), can initiate hyperglycemia. The reflex increase in blood glucose concentration thus can compensate for the impaired cerebral blood flow

adrenergic influence on the β -cell in this reflex, whereas adrenal catecholamines are of minor importance. This relative unimportance of the adrenal glands in the readjustments to stress has also been shown in a recent study by Bloom, Edwards & R. (1977). They found that only small amounts of catecholamines were released from the adrenal medulla even during severe hypoxia in the young conscious calf.

Our finding that direct sympathetic nerve fibres can play an important role in the control of endocrine pancreas is supported by previous experiments in which the splanchnic nerves or the mixed autonomic pancreatic nerve have been stimulated electrically (Bruilzen & Howell 1970, Marliss *et al.* 1972, 1973, Bloom, Edwards & Vaughan 1973, *et al.* 1973, Kaneto, Kajinuma & Kosaka 1975, Bloom & Edwards 1975, see also review by Woods & Porte 1974). These studies have shown, in general, that activation of sympathetic nerves leads to a glucose and glucagon release but a roughly maintained insulin release. The relative maintenance of the insulin levels despite hyperglycemia has been interpreted as an α -adrenergic inhibition of the normal glucose-induced insulin release. However, Kaneto and collaborators (1975) reported that the slight increase in insulin release in response to splanchnic nerve stimulation in the dog was abolished by pretreatment with propranolol, suggesting that the sympathetic nerves may influence both the α - and β -receptors of insulin-producing cells. However, direct electrical activation is an unphysiological stimulus since it will activate both afferent and efferent axons belonging to the adrenergic, cholinergic, and, perhaps, peptidergic system. Hence, the observed effects of electrical stimulation on insulin release will comprise the net sum of these different neurogenic influences on the β -cell. The present finding of a clear-cut decrease in the insulin concentration in portal blood may thus represent the true reflex adrenergic response of the β -cell, even if one can speculate that there exist selective nerve fibres which terminate on the α - or the β -receptors and that such fibres might be independently engaged during different reflexes.

The present investigation demonstrated that unloading of the carotid baroreceptors led to a 3-fold increase in the glucagon concentration and a 50 per cent decrease in the insulin concentration of portal blood. These changes indicate an enhanced release of glucagon and an inhibited release of insulin from the pancreas, providing the portal blood flow was concomitantly changed by the baroreceptor reflex. Portal blood flow was not recorded in the present investigation, but previous studies on cats have demonstrated that the blood flow through different splanchnic organs, such as the small intestine and liver, remains virtually unchanged during complete unloading of the carotid baroreceptors, since the increase in vascular resistance is moderate and counterbalanced by the concomitant increase in perfusion pressure (Öberg 1964, Greenway, Lawson & Mellander 1967). Furthermore, the adrenergic vasoconstriction in splanchnic organs shows a characteristic "autoregulatory escape" phenomenon, implying that the vessels will dilate despite continuous adrenergic stimulation (Folkow *et al.* 1964, Greenway, Lawson & Mellander 1967). If pancreatic blood flow is mechanically reduced it seems that the insulin secretion is impaired only when pancreatic blood flow is reduced to 50% of its normal value for 40 min (Rappaport *et al.* 1971). It should also be noted that glucagon and insulin changed in opposite directions. It seems likely that the concentrations of the two hormones would have changed parallel to each other if the effects were merely caused by changed portal blood flow. It follows

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so that the delivery of glucose to the brain remains relatively unchanged in these states. The relay station in the brain for this metabolic reflex has not been settled, but a test structure is the ventromedial part of the hypothalamus. Stimulation of this region has been shown to elicitate hyperglycemia, hyperglucagonemia and hypoinsulinemia (e.g. D 1953 Folkow & Euler 1954 Frohman & Bernardis 1971 Müller Frohman & C 1973 Frohman Bernardis & Stachura 1974).

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Differential effect on the sympathetic transmitter level in uterus and other organs of guinea-pig by drugs interfering with adrenergic nerve functions

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Abstract

THORBERT, G., ALM, P., OWMAN, CH., ROSENGREN, E. and SJÖBERG, N.-O. *Differential effect on the sympathetic transmitter level in uterus and other organs of guinea-pig by drugs interfering with adrenergic nerve functions.* Acta physiol. scand. 1978. 104. 203-21.

Uterine adrenergic blocking agents with different modes of action were administered and the rate of norepinephrine depletion was studied in different uterine regions of guinea-pig, and compared with that in the adrenal and submandibular gland by a combination of fluorescence histochemical and spectrofluorometric methods. The tyrosine-hydroxylase inhibitor, α -methyl- β -methyl-L-tyrosine, as well as reserpine and guanethidine, produced a significant reduction in uterine norepinephrine in the heart and submandibular gland, but not in the uterus. Similar action on the sympathetic transmitter, though less clearcut, was seen with 6-hydroxydopamine. Norepinephrine-depleting effect of reserpine did not distinguish between those adrenergic nerves innervating the uterus from those of the control tissues. It is suggested that the differential effects may be related to lower activity in the uterine adrenergic nerve system, probably because this system is to a large extent composed of short adrenergic neurones, which appear to have particularly slow transmitter turnover.

Uterine adrenergic innervation undergoes dramatic alterations during pregnancy and shows a unique susceptibility to sex steroids as reflected in changes of the transmitter level (Owman, Sjöberg and Sjöstrand 1974, Owman and Sjöberg 1976). In order to investigate the structural and biochemical mechanisms underlying these changes, the guinea-pig has for several reasons been chosen as a suitable experimental animal model. On the basis of denervation studies, using histochemical and chemical analyses of the neurotransmitter the original distribution of the uterine adrenergic innervation of the guinea-pig have been described in detail (Thorbert *et al.* 1977). The marked variation in the transmitter level occurring during pregnancy and in the post partum period has been followed during narrow time intervals (Owman *et al.* 1975, Thorbert *et al.* 1978 a, Gårdmark, Owman and Sjöberg 1971), and it has been possible to relate to alterations in transmitter synthesis as well as to degenerative and regenerative phenomena in the local innervation. These peculiar properties of the uterine adrenergic innervation may at least partly be related to the susceptibility of the nerves to the action of sex steroids (*cf.* Thorbert, Alm and Rosengren 1978 b).

In the present paper certain functional properties of the uterine adrenergic arm have been further elucidated by drugs known to interfere with various aspects of adrenergic function.

Materials and methods

Animals. Non-pregnant, sexually mature virgin guinea-pigs of the Dunkin-Hartley strain (200 b.w.t.) were used. They were housed under standard conditions with regard to temperature, light and light and were given pellets (Astra Ekos, Sweden) and water *ad lib*. The oestrous cycle was determined by daily inspection of the vaginal orifice. A smear was taken when the vagina was open. The smears were classified according to Sjövall (1938). The day of maximal cornification, before the influx of keratin in the smear, was called day 1 of the cycle. The animals were killed on days 8-12. An additional necropsy was taken on the day of sacrifice and only animals with a clear leucocytic dominance were accepted for study.

In vivo experiments. The animals received the following drugs: 1144/68 (methyl ester of α -methyl tyrosine, Hälske Sweden), dissolved in saline, was given *i.p.* in a dose of 500 mg/kg followed by a second injection 8 h later. The animals were sacrificed 24 h after the first injection. Reserpine (Geigy) (diluted in 0.1 M citric acid) was given *i.p.* in doses of 0.1 and 0.01 mg/kg. The animals were killed 24, 48 and 96 h after the administration. Guanethidine bisulphate (Hälske Sweden) was dissolved in saline. The amount administered was calculated as free base and was given in an *i.p.* dose of 10 mg/kg. The animals were studied 6, 12 and 48 h later. Metaraminol bitartrate, dissolved in saline and calculated as free base, was given *i.p.* in a dose of 10 mg/kg, which was followed by another injection 4 h later. The animals were studied 12, 24 and 36 h after the first injection. 6-Hydroxydopamine (2,4,6-trihydroxyphenylamine hydrochloride = 6-OH DA) dissolved in ice-cold saline containing ascorbic acid (0.2 mg/ml), was given *i.p.* under light ethyl ether anaesthesia into the saphenous vein in doses of 5, 10 and 20 mg/kg. The animals were studied 4 h later.

The animals were sacrificed under a light ethyl ether anaesthesia at the various time intervals after drug administration, and tissue pieces were taken from the following organs: submandibular gland (auricle—mainly left), and uterus (specimens from the suspensory ligament and the mid-portion of the uterine horn, as well as from the cervix).

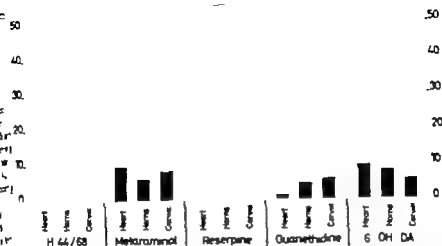
Neuronal noradrenaline was demonstrated histochemically by the fluorescence technique of Falck & Hillarp (for details, see Björklund, Falck and Owman 1972).

The fluorimetric method of Bartlett & L (1938), as modified by Häggendal (1963), was used for the quantitative chemical determinations of the amine in preparations consisting of the entire heart, uterus and cervix. The recovery of noradrenaline was 83 ± 3 (mean \pm SE, $n=7$). Correction for recovery was not made. The coefficient of variation was 3% for a pool of uterine tissue ($n=5$) and 5% for a pool of cardiac tissue ($n=5$). Because of a possible occurrence of α -methylated metabolites from α -methyl tyrosine in experiments where 1144/68 was given, the detectability of such metabolites (α -methyl dihydroxyphenylalanine, dopamine and noradrenaline) was tested by adding these α -methylated compounds to tissue homogenates with known endogenous noradrenaline content. In no case were the fluorimetric readings significantly changed ($p > 0.05$) by the addition of the α -methylated compounds.

Statistics. It was found that the recorded noradrenaline values after the drug treatments and in the animals were beset with heterogeneous variation as tested by Bartlett's test (Snedecor 1946). To transform the measured noradrenaline values into their logarithms, variance heterogeneity was found to be non-significant. The transformed values were treated statistically in the analysis of variance using factorial coefficients (Snedecor 1946).

In vitro experiments. Tissue slices were prepared according to Hamberger (1967) from myometrium of the uterine horns and from myocardial tissue. Krebs-Ringer bicarbonate buffer containing 8 g/l glucose, was used as incubation medium. The composition of the Krebs-Ringer medium was described by Hamberger (1967). The buffer was saturated with 95% O_2 and 5% CO_2 , and the incubation flasks were gassed every 60 min. In the experiments with 6-OH DA the incubation buffer was also treated with ascorbic acid (0.02 mg/ml).

The slices were collected in ice-cold incubation buffer immediately following dissection. They were started by incubating the tissue slices in drug-free buffer for 20 min at 37°C to equilibrate the slices to this temperature, which was then used in the further incubation procedure. 6-OH DA, reserpine, guanethidine or metaraminol (see also above) were added to the incubation medium in 10^{-4} – 10^{-5} M.



Effect of various drugs on the total organ content of noradrenaline in the heart, the two uterine horns and the cervix of guinea-pigs. Mean noradrenaline content after the various treatments expressed as percentage of the mean value from 5 untreated, drug-free controls is shown. The amount of drug given, injection route used, and the time interval between the (first) injection and sacrifice are for the various drugs: H 44/68: 500 mg/kg *i.p.* twice, with 8 h interval, 24 h. Metaraminol: 10 mg/kg *s.c.* twice with 4 h interval, 12 h. Reserpine: 10 µg/kg *i.p.* once, 12 h. Guanethidine: 30 mg/kg *i.p.* once, 6 h. 6-OH-DA: 50 mg/kg *s.c.* once, 4 h. Analyses of variance using factorial coefficients showed significant differences at $p < 0.05$ in the degree of noradrenaline reduction in heart vs. horns or cervix following treatment with H 44/68, reserpine, and guanethidine. Other comparisons gave no statistical significance.

traces, and the tissue slices are incubated in the presence of the respective drug for 10–120 min. Incubation time and drug concentration was tested on material from 4 animals, and the various results also included control tissues incubated in drug-free medium only. Immediately after the incubation was finished (in the case of metaraminol slices as always followed by rinsing for 30 min in drug-suspension buffer), the tissue slices were frozen in propen-propylene cooled by liquid nitrogen, and stored according to the method of Pakk and Hållarp (see Björklund *et al.* 1972) for fluorescence histochemistry. From every animal used, specimens from the myocardium and the uterus are also taken for fluorescence histochemistry without previous incubation.

Results

Effects *in vivo* Treatment with the tyrosine hydroxylase inhibitor H 44/68, during a 16-hour period resulted in a marked decrease in the number of the sympathetic nerves in the submandibular gland and the heart. The remaining nerve terminals exhibited a moderately decreased fluorescence. The effect of H 44/68 on the uterine adrenergic innervation was much less pronounced. This was reflected in the chemical determinations of noradrenaline (Fig. 1), showing that 42 per cent of the total amount remained in the cervix and uterine horns and 22 per cent in the heart after this treatment. Statistical analysis showed that the effect on the heart was more pronounced than in the uterus ($p < 0.05$ Fig. 1).

Effects *in vivo* The result of the administration of 10 mg/kg twice with 4 h interval was studied 12, 24 and 36 h after the first injection. Only very few adrenergic nerves with a slight

fluorescence were seen at all time intervals, and the findings were the same in all examined.

In accordance with the fluorescence histochemical observations, tissue noradrenaline markedly reduced to a similar extent in all preparations analyzed fluorimetrically (Fig. 1).

Reserpine

Effects in vivo Changes in the neuronal level of noradrenaline were examined at various time intervals following blockade of the amine storage mechanism by reserpine (10 µg). In the auricle and submandibular gland a marked decrease in the number of green-luminescent nerves was seen already after 6 h, and only very few weakly fluorescent nerves were seen 6 h later. At 24 h the fluorescence had disappeared in all nerves of these two structures. Fluorescence in the nerves returned progressively, as seen 48 and 96 h after the injection, at 96 h almost a normal number and fluorescence intensity had been attained.

The reduction in the transmitter followed the same time-course in the uterus, but levels were clearly higher than in the auricle and submandibular gland except 24 h after injection when virtually no fluorescent nerves were seen (only in the uterine suspensory ligament did a substantial number of fluorescent nerves remain at this time interval). Accordingly, a nearly complete restitution of the fluorescent network of adrenergic nerves was seen already within 48 h after the injection.

The difference in reserpine sensitivity could be confirmed in the chemical determinations in which the noradrenaline reduction produced by a dose of 10 µg/kg at 12 h was significantly more pronounced in the heart than in the tissues from the reproductive tract (Fig. 1).

Effects in vitro Incubation of myocardial and myometrial tissue in the presence of 10^{-4} and 10^{-5} M reserpine resulted, after 60 or 120 min, in a marked reduction in the level and number of fluorescent nerves. The extent of the reduction was the same in both tissues. The yellowish fluorescence produced by reserpine in the tissue did not interfere with microscopic evaluation of the specific fluorescence in the adrenergic nerves.

Guanethidine

Effects in vivo Guanethidine is an adrenergic neuron-blocking agent which produces transmitter depletion from the postganglionic sympathetic nerves. Thus, 2 h after the injection there was a moderate reduction in the fluorescence of the adrenergic nerves in the submandibular gland and the heart auricle, whereas the nerve fluorescence in the uterus seemed unaffected. At later time intervals (6–24 h after the injection) no or only few fluorescent adrenergic nerves remained visible in the auricle, submandibular gland, and uterine horns and cervix. The effect on neuronal noradrenaline was much less pronounced in the uterine suspensory ligament. The chemical determinations of noradrenaline showed a similar pattern of difference in the response to guanethidine, and factorial analysis showed that the amount of transmitter reduction in the reproductive tract was significantly smaller than that in the heart ($p < 0.05$, Fig. 1).

Effects in vitro Incubation for 60 or 120 min in the presence of 10^{-4} – 10^{-5} M guanethidine

tered the adrenergic nerve fluorescence in both myometrial and myocardial tissue the seemed to be more pronounced in the latter tissue.

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is in vivo Most of the preparations taken for fluorescence microscopy were obtained animals receiving 20 mg/kg of 6-OH-DA. In the aortic and submandibular gland was a prominent general decrease in the intensity of nerve fluorescence; remaining were barely visible. The fluorescence microscopic picture was different in the uterine and cervix: only a very small number of fluorescent nerves remained, but with a distinctly higher fluorescence intensity than in the cardiac nerves. The chemical determinations showed a marked reduction in tissue noradrenaline. The extent of reduction was not significantly different when comparing the different tissues (Fig. 1).

in vivo Doses of 6-OH-DA (10 and 5 mg/kg) were also tested. The reduction in tissue noradrenaline was proportionally less marked at these dose levels. There was a tendency to less extensive relative fall in the transmitter of the uterine horns (cervix not measured) compared to the heart, though the difference was not statistically significant.

in vitro Ten minutes incubation with 10^{-6} M of 6-OH-DA slightly reduced the fluorescence intensity of the adrenergic nerves in the heart and uterine tissue. After 10 and 30 min incubation almost no adrenergic nerves could be seen. A lower dose (10^{-7} M) of 6-OH-DA produced essentially the same changes, although less pronounced. The myocardial adrenergic nerves seemed slightly more susceptible than the myometrial nerves to the action of 6-OH-DA.

Discussion

The female reproductive tract of the guinea-pig receives a complex supply of adrenergic nerves (Thorbert *et al.* 1977), originating both in classical prevertebral sympathetic ganglia and in peripheral relays located in the uterovaginal region (Frankenhäuser's ganglia or plexus). The functionally unique properties of this innervation (Owman *et al.* 1974, Owman & Sjöberg 1976) have been elucidated in pharmacological experiments in which the sympathetic nerves of the heart (auricular tissue) have been chosen as reference. The effect of steroids (Thorbert *et al.* 1978 b) and pregnancy (Owman *et al.* 1975, Thorbert *et al.* 1978 a) on the uterine nerves of guinea-pig has recently been investigated in detail. In the present paper certain aspects of adrenergic neurotransmission have been studied using drugs that (a) block amine synthesis (H44/68) and (b) storage (reserpine), that (c) release transmitter by different processes (metaraminol and guanethidine), and (d) produce nerve degeneration through chemical sympathectomy (6-OH-DA).

The hydroxylation of tyrosine to dihydroxyphenylalanine is the rate-limiting step in the synthesis of noradrenaline (Levitt *et al.* 1965). *o*-Methyl-*p*-tyrosine is a potent inhibitor of tyrosine hydroxylase (Nagatsu, Levitt and Udenfriend 1964), and produces a marked reduction in tissue noradrenaline (Spector, Sjoerdsma and Udenfriend 1965). This inhibitor or its methyl-ester H44/68 (Corrodi and Hanson 1966, Corrodi and Malmfors 1966) has been extensively used to estimate the noradrenaline turnover under various conditions (*cf.* Brodie *et al.* 1966). In the present study treatment with H44/68 resulted in a marked reduction in

fluorescence were seen at all time intervals, and the findings were the same in all examined.

In accordance with the fluorescence histochemical observations, tissue noradrenaline markedly reduced to a similar extent in all preparations analyzed fluorometrically (Fig. 1).

Reserpine

Effects in vivo Changes in the neuronal level of noradrenaline were examined at various time intervals following blockade of the amine storage mechanism by reserpine (0.1 mg). In the auricle and submandibular gland a marked decrease in the number of green-fluorescent nerves was seen already after 6 h, and only very few weakly fluorescent nerves were seen 6 h later. At 24 h the fluorescence had disappeared in all nerves of these two structures. Fluorescence in the nerves returned progressively as seen 48 and 96 h after the injection: at 96 h almost a normal number and fluorescence intensity had been attained.

The reduction in the transmitter followed the same time-course in the uterus, but levels were clearly higher than in the auricle and submandibular gland except 24 h after injection when virtually no fluorescent nerves were seen (only in the uterine suspensory ligament did a substantial number of fluorescent nerves remain at this time interval). Accordingly, a nearly complete restitution of the fluorescent network of adrenergic nerves was seen already within 48 h after the injection.

The difference in reserpine sensitivity could be confirmed in the chemical determination in which the noradrenaline reduction produced by a dose of 10 µg/kg at 12 h was significantly more pronounced in the heart than in the tissues from the reproductive tract (Fig. 1).

Effects in vitro Incubation of myocardial and myometrial tissue in the presence of 10^{-4} M reserpine resulted, after 60 or 120 min, in a marked reduction in the level and number of fluorescent nerves. The extent of the reduction was the same in both tissues. The yellowish fluorescence produced by reserpine in the tissue did not interfere with microscopic evaluation of the specific fluorescence in the adrenergic nerves.

Guanethidine

Effects in vivo Guanethidine is an adrenergic neuron-blocking agent which produces transmitter depletion from the postganglionic sympathetic nerves. Thus, 2 h after the injection there was a moderate reduction in the fluorescence of the adrenergic nerves supplied to the submandibular gland and the heart auricle, whereas the nerve fluorescence in the uterus seemed unaffected. At later time intervals (6–24 h after the injection) no or only few fluorescent adrenergic nerves remained visible in the auricle, submandibular gland, and in the uterine horns and cervix. The effect on neuronal noradrenaline was much less pronounced in the uterine suspensory ligament. The chemical determinations of noradrenaline showed a similar pattern of difference in the response to guanethidine, and factorial analysis showed that the amount of transmitter reduction in the reproductive tract was significantly smaller than that in the heart ($p < 0.05$, Fig. 1).

Effects in vitro Incubation for 60 or 120 min in the presence of 10^{-4} – 10^{-6} M guanethidine

and the adrenergic nerve fluorescence in both myometrial and myocardial tissue the reduction seemed to be more pronounced in the latter tissue.

DA

In vivo Most of the preparations taken for fluorescence microscopy were obtained from animals receiving 20 mg/kg of 6-OH-DA. In the auricle and submandibular gland there was a prominent general decrease in the intensity of nerve fluorescence; remaining fluorescence was barely visible. The fluorescence microscopic picture was different in the uterus and cervix, only a very small number of fluorescent nerves remained, but with a distinctly higher fluorescence intensity than in the cardiac nerves. The chemical determinations showed a marked reduction in tissue noradrenaline. The extent of reduction was not significantly different when comparing the different tissues (Fig. 3).

Lower doses of 6-OH-DA (10 and 5 mg/kg) were also tested. The reduction in tissue noradrenaline was proportionally less marked at these dose levels. There was a tendency to a more extensive relative fall in the transmitter of the uterine horns (cervix not measured) compared to the heart, though the difference was not statistically significant.

In vitro Ten minutes incubation with 10^{-4} M of 6-OH-DA slightly reduced the fluorescence intensity of the adrenergic nerves in the heart and uterine tissue. After 10 and 30 min incubation almost no adrenergic nerves could be seen. A lower dose (10^{-5} M) of 6-OH-DA produced essentially the same changes, although less pronounced. The myometrial adrenergic nerves seemed slightly more susceptible than the myocardial nerves to the action of 6-OH-DA.

Discussion

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neuronal noradrenaline, the effect being more pronounced in the heart and submandibular gland than in the uterine tissue. Hence this may be due to a difference in the turnover of the total transmitter pool in terms of, for instance, a lower noradrenaline release or a more efficient transmitter re-uptake in the nerves of the uterine tissue. A similar effect has been shown for the effect of H44/68 in male rats, in which the noradrenaline remained essentially unchanged in the vas deferens and seminal vesicle while a pronounced reduction was seen in the heart and submandibular gland (Swedin 1970). The smooth musculature of the male internal genital organs are supplied entirely with short adrenergic neurons (Sjöstrand 1965); this class of nerves forms part of the innervation in the female uterus (Thorbert *et al.* 1977). Hence, a lower rate of turnover in the system of short adrenergic neurons may have contributed to the differential effect of H44/68 in the present experiments.

α -Methyl-*p*-tyrosine is formed from the methyl-ester H44/68, *in vitro* (Corrodi and Carlsson 1966), and is then partly transformed into α -methyl-noradrenaline via α -methyl-dihydroxyphenylalanine and dopamine (Maltre 1965; Carlsson, Miesch and Nil 1968). The fluorescence yield of these α -methylated compounds in the formaldehyde fluorescence method (cf. Björklund *et al.* 1972) is approximately the same as that of noradrenaline. This would mean that, if anything, the fluorescence microscopic evaluation of the degree of reduction in neuronal noradrenaline after treatment with H44/68 may have been underestimated. On the other hand, it was found that addition of these α -methylated products to tissue homogenates does not interfere with the fluorimetric determination of noradrenaline. Another aspect of this biotransformation of H44/68 is that α -methyl-noradrenaline may cause a depletion of authentic noradrenaline from the sympathetic nerves. Such depletion of noradrenaline from the sympathetic nerves was obtained with metaraminol which acts by displacement of noradrenaline from its storage sites after uptake into the nerve (Andén 1964). This mechanism of action did not distinguish between the adrenergic nerves in the uterine tissue on one hand and in the heart and submandibular gland on the other. This was confirmed in the chemical determinations showing an approximately equal depletion of noradrenaline in both types of tissues. It is therefore concluded that noradrenaline depletion by α -methylated metabolites has not been the major mechanism in the experiments utilizing H44/68.

It is generally accepted that the main effect of reserpine involves blockade of the ATP dependent uptake and storage mechanism of the amine granules (see Carlsson 1975). As a net result of the normal activity of the neuron, the noradrenaline level is reduced as new storage granules are synthesized (Häggendal and Dahlström 1971; Giachetti and Sjöstrand 1975). This means that experiments with reserpine provide a kind of indirect measure of neuronal activity in the sympathetic system (Gillespie and McGrath 1974). According to reserpine treatment produced the same pattern of differential noradrenaline reduction in the heart-submandibular gland as H44/68. The experiments confirm a similar observation on the female reproductive tract of rabbits (Nilsson 1964; Owman and Sjöstrand 1967) that the interruption of the postganglionic adrenergic nerves in the uterus results in the rate of reduction in tissue noradrenaline, whereas stimulation of the hypogastric plexus had the opposite effect. The male internal genital organs are also markedly resistant to the action of reserpine (Sjöstrand and Swedin 1967; Dall and Evan 1974), which, in accordance

the reasoning above, would support the view that the contribution of short adrenergic nerves may be responsible for a slower turnover of noradrenaline in the uterus compared with sympathetically innervated organs.

methidine is a sympatholytic agent which, in acute experiments, produces adrenergic blockade and also has a direct noradrenaline-depleting effect (*cf* Costa 1966, Abbas Dodd 1974). This type of interference with sympathetic nerve function also showed a distinctly more pronounced effect in the heart tissue than in the uterus. There may be several explanations for this differential effect, one being that the noradrenaline pool available for immediate transmitter release is relatively smaller in the short adrenergic neurons (Orden 1969, Swedin 1970, 1972).

6-OH-DA produces a chemical sympathectomy through a specific neurotoxic action on nerve terminals (Thoenen and Tranzer 1968). Under the conditions applied in present experiments, 6-OH-DA markedly reduced the noradrenaline content in all tissues analyzed. It is notable that the guinea-pig seems to be more resistant to this neuroamine than other commonly used laboratory animals such as the rat and rabbit (unpublished observations). Our data on 6-OH-DA effects on the guinea-pig heart are consistent with data presented by others (O'Donnell and Saar 1974, Bentley, Shibata and Cheng 1975). With the lower doses there was a tendency (though not statistically significant) to a more pronounced effect in the cardiac tissue, and fluorescence microscopy showed a slightly greater appearance of remaining fluorescent nerves in the uterus and heart. Whether this reflects a different susceptibility in various types of sympathetic neurons is not possible to judge on the basis of the present material. It is notable, though, that noradrenaline in the deferens is relatively resistant to the action of 6-OH-DA compared to other sympathetically innervated organs (Jocsson and Sachs 1970), unless this is given in high, repeated doses (Cottle and Nash 1974).

The extensively developed smooth musculature in the entire system of internal male genital organs is innervated by the special type of "short adrenergic neurons" (Sjöstrand and Öwman, Sjöberg and Sjöstrand 1974, Baumgarten, Öwman and Sjöberg 1975), which reside in the peripheral ganglia located adjacent to the effector organ. This predominant supply by short adrenergic neurons in the male made it possible to conveniently analyze pharmacological and physiological differences between these and the "classical" sympathetic nerves, which arise in para- and prevertebral ganglia. Using a number of approaches it has been found that the short adrenergic neurons constitute a functionally unique system of nerves with several peculiarities recently listed and discussed by Baumgarten *et al.* (1975). This kind of short adrenergic neurons represents a considerable contribution to the sympathetic innervation of the guinea-pig uterus (Thorbert *et al.* 1977). The presently observed pharmacological differences in comparison with tissues innervated by conventional sympathetic nerves resemble—though they seem to be quantitatively less marked—those found in the male genital organs, and probably reflect this contribution of short adrenergic nerves to the uterus. Another possibility is that the entire sympathetic innervation as such is peculiar to the uterus, an organ with several properties that are fundamentally different from other sympathetically innervated organs (*cf* Spaziani 1976). Taking the results from the pharmacological experiments together, they seem to indicate that the net rate of transmitter turnover

is comparatively low in the uterine adrenergic innervation including such transfer properties as, e.g. release of only comparatively small transmitter quantities (cf. S. 1971). This assumption is supported by the results obtained in the incubation experiments in which the pharmacological differences were to a large extent eliminated, namely in conditions when the postganglionic innervation in the uterine preparations had been separated from its normal functional connections.

A factor that might have been of importance in determining the pharmacological effects on the various tissues examined is the blood flow through the organs, which may have influenced the tissue concentration of the drugs tested. There are several reasons to believe that blood flow differences have not been a primary factor in the present experiments: (1) the drugs have usually been given in amounts that are well above the minimum effective dose, (2) the difference in response between uterus on one hand and heart and submaxillary gland on the other was not a uniform outcome, metaraminol and 6-OH DA both have specific receptors for reasons discussed above, and (3) there is evidence that the flow rate in the uterus is intermediary to those of the heart and salivary gland (Kopm, Gordon and Fischer 1965; Fischer, Kopin and Wortman 1964).

It is thus concluded from the various experiments that the adrenergic nerves of the uterus have a low functional activity in the nonpregnant guinea-pig. One probable reason for this is that the short adrenergic neurons, with their slow turnover rate, form a prominent contribution.

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Effect of lowering of the temperature on the tissue levels of cAMP, cGMP, PGE and $\text{PGF}_{2\alpha}$ in spontaneously beating rat atria preparation

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Abstract

PARANTAINEN, J., T. METSÄ-KETELÄ and H. VAPAAHALO: Effect of lowering of the temperature on the tissue levels of cAMP, cGMP, PGE and $\text{PGF}_{2\alpha}$ in spontaneously beating rat atria preparation. *Acta physiol. scand.* 1978. 104. 213-216.

The levels of cAMP, cGMP, PGE, and $\text{PGF}_{2\alpha}$ are measured in spontaneously beating rat atria preparation at 20°C and 37°C. At 20°C the formation of cAMP increased in correlation with the markedly increased stroke and reduced frequency. The other biochemical parameters are not significantly affected by the temperature.

Key words: Cyclic nucleotides, prostaglandins, isolated rat atria, temperature.

Changes in the bath temperature alter the function of isolated heart preparations substantially. Cold environment slows down the heart rate and increases the amplitude, while raising of the temperature has the opposite effect. These adaptive changes seem to be mainly compensatory and the work output of the heart (frequency \times amplitude) remains almost the same. Knoch and Szecivanyi (1968) claim that the adrenergic receptor function of the atria alters from β to α type as the bath temperature is lowered. Amer and Byrnes (1975) state these receptor alterations in the tissue levels of cAMP and cGMP. Because cyclic nucleotides and prostaglandins (PG) are anatomically and functionally interrelated in many tissues, we have measured both these biochemical parameters in relation to temperature alteration of spontaneously beating rat atria preparation.

Material and methods

Male Sprague-Dawley rats weighing 250-270 g were killed by dislocation of the neck, their hearts were removed, atria excised, and the preparations were made as previously, oxygenated Tyrode solution containing 1 000 IU/l heparin (Medica Ltd, Helsinki). The atria were incubated in 20 ml oxygenated bath con-

The results have been presented in before (Parantainen *et al.* 1977).

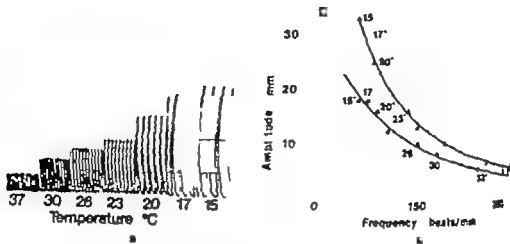


Fig. 1. Lowering of the bath temperature markedly decreases the heart rate and increases the amplitude. The changes are mutually compensatory and the work performed by the atria remains the same (A). ● are recordings from two different preparations.

continuous flow of Tyrode (1.5 ml/min). Four preparations were installed parallelly and the aortic responses registered isometrically by a Harvard Apparatus physiograph N°o 150. The isometric force was 0.5 g. After the stabilization of the preparation to 37°C or 20°C was completed (usually about 5 min) the preparation was removed, clamped with tongs precooled in liquid nitrogen and frozen in liquid nitrogen with Brau Microdisseminator. The pulverized tissue was dissolved in 500 µl of 30% TCA for determination of PGs, or correspondingly to 5% TCA for cyclic nucleotide assay. The supernatant was heated at 95°C for 1 min and stored at -20°C for analysis. cAMP and cGMP are measured by protein binding methods of Gilman (1970) and Dunne et al. (1974), respectively. PGE (counted as 3H) and PGF_{2α} were determined by specific radioimmunoassays (Gershman *et al.* 1972, Jaffe and Behrens 1971, Jaffe *et al.* 1971, Levine and Van Vunakis 1970). Student's *t*-test was used in statistical comparisons.

Results

Lowering of the bath temperature to 20°C decreased the heart rate and caused a substantial increase in the amplitude (Fig. 1 a). The mechanical changes were mutually compensatory, the work performed by the preparation remaining the same at different temperatures (Fig. 1 b). The biochemical findings are summarized in Table I. Significant differences were obtained in the formation of cAMP. Compared to the level at 37°C (5.24 pg/mg protein)

TABLE I. Tissue levels of cAMP, cGMP, PGE and PGF_{2α} at tested temperatures.

	37°C		20°C		<i>t</i> -test
	\bar{x}	S.E. (n)	\bar{x}	S.E. (n)	
cAMP ^a	5.24 ± 0.44	(8)	6.55 ± 0.43	(7)	< 0.05
cGMP ^a	0.41 ± 0.03	(8)	0.36 ± 0.06	(8)	N.S.
PGE ^b	20 ± 3	(6)	23 ± 7	(8)	N.S.
PGF _{2α} ^b	7 ± 10	(4)	31 ± 4	(4)	N.S.

^a pg/mg protein.
^b pmol/mg protein.

formation of this nucleotide increased by 25% when the temperature was lowered to 20°C (0.5). The formation of cGMP did not change. Neither were there any significant alterations in the tissue levels of PGE or PGF_{2α}.

Discussion

alterations in mechanical work performed by the atria at different temperatures are small and, correspondingly should imply major changes in the biochemistry that rates the inotropic and chronotropic action. Yet no such changes were detected with regard to cyclic nucleotides and PGs. The only significant finding was the elevation of cAMP which paralleled the increase in amplitude when the temperature was lowered. This is in accordance with the finding that cAMP mediates the positive inotropic action in adrenergic stimulation (Robbion *et al.* 1965; Vapalahti *et al.* 1978). Accordingly Wollenberger *et al.* (1971) in ventricles of frog heart, and Brooker (1973) in isolated strips of frog heart demonstrated that in spontaneously contracting muscle the positive inotropic action was associated with an increase in tissue cAMP concentration. The Wollenberger group also revealed that rising of tissue cGMP may accompany the rise in cAMP. Similar cAMP-cGMP interaction was observed in one series of our experiments with significant lowering of cGMP. However, the positive inotropic reaction may also be independent of cAMP (Oye and Langset 1979).

The relative insensitivity of PG formation to temperature changes was unexpected, as PG-synthetase, the enzyme system that generates PGs, is reported to be sensitive to temperature (Arai *et al.* 1973). Furthermore, PGs are known to be able to modulate temperature regulation in some smooth muscle preparations (Strong and Chandler 1972) and they have had a capacity for regulation of local blood flow. Compensatory mechanisms may be involved in maintaining the relative stability of the tested biochemical parameters. The adaptation of heart muscle to different temperatures seems to depend on other systems, the possible candidates being other ATP-dependent enzymes, high-energy fatty acids and regulatory systems within the cell.

In conclusion, amongst the tested parameters only cAMP tended to parallel changes in work output at different temperatures. The impetus for this correlation remains to be elucidated.

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Activity of acid hydrolases in skeletal muscle of untrained, trained and detrained mice of different ages

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Abstract

ÖM, L., V VIHKO, E. ÅSTRÖM and A. U. ARSTILA. *Activity of acid hydrolases in skeletal muscle of untrained, trained and detrained mice of different ages.* Acta physiol. scand. 1978. 104. 217-224.

Activities of p-nitrophenylphosphatase, β -glucuronidase and β -N-acetylglucosaminidase from crude muscle homogenates of 4 and 7 months old mice were assayed after short-term intensive and long-term moderate training and after terminated training. In the older untrained mice the activity of the hydrolases was higher than in the younger mice. The level increased with training and this increase was far more marked in the older animals. Cessation of training for 7 and 21 days decreased the activity in the muscle but was again decreased 42 days later and close to the level observed in the trained mice. In mice 3 days terminated training increased the activity of the acid hydrolases above the level of the 4 months but after additional 4 and 11 days' terminated training the activity decreased to slightly that of the trained mice. The changes were most prominent in the activity of β -glucuronidase and to a extent in that of β -N-acetylglucosaminidase while p-nitrophenylphosphatase activity was almost unaffected by training or terminated training. The effects of terminated training can be interpreted as represented catabolic processes in the turn-over of tissue components of skeletal muscle.

Key words: Acid hydrolases, age, detraining, skeletal muscle, training.

Activity of acid hydrolases in skeletal muscle occur normally and increased levels are often reported in pathological conditions (Kar and Pearson 1972, Welstock and Iodice 1973). As their normal activity most probably contributes to regulating normal turn-over of fiber contents, increased activity could be expected during periods of physical exercise. It has been demonstrated that physical training induces higher activity of β -glucuronidase in the skeletal muscle of mice, but does not significantly affect the activities of p-nitrophenylphosphatase or β -N-acetylglucosaminidase (Viikio *et al.* 1974). However this observation was made on a rather small sample (five or six animals per compared groups), and in order to study the effect more carefully the present investigation was undertaken. The investigation was performed with two types of training (intensive short-lasting and moderate

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I. Effect of age on the activities of acid hydrolases in skeletal muscle of mice

tissue basis	Enzyme	Activity		Difference t between groups	t	P
		Younger	Older			
mean	p-NPPase	6.456 ± 0.091 (27)	9.165 ± 0.159 (30)	42.0	14.3367	<10 ⁻⁴
	β-GUase	0.038 ± 0.002 (27)	0.102 ± 0.005 (30)	75.9	7.6243	<10 ⁻⁴
	β-GAase	1.599 ± 0.038 (27)	2.401 ± 0.081 (30)	50.2	8.6101	10 ⁻⁴
wt-weight	p-NPPase	1.026 ± 0.021 (27)	1.149 ± 0.022 (30)	12.0	4.0377	<0.0005
	β-GUase	9.218 ± 0.396 (27)	11.561 ± 0.565 (30)	3.2	2.7786	0.01
	β-GAase	0.255 ± 0.007 (27)	0.303 ± 0.011 (30)	18.8	3.5447	<0.001

total activities of acid p-nitrophenylphosphatase (p-NPPase), β-glucuronidase (β-GUase) and β-N-acetylglucosaminidase (β-GAase) in crude muscle homogenates of young (approx. 4 months) and older (ca. 7 months) mice. The activities are expressed as nmoles p-nitrophenol formed per mole either per protein or per mg tissue wet-weight, except for β-GUase per wet weight here the activity is pmoles d of nmoles. The figures are mean values ± S.E. and the number of animals per group is given in brackets. For further details: See Material and methods.

II. Effect of training on the activities of acid hydrolases in skeletal muscle of mice

tissue basis	Enzyme	Age (months)	Activity		Increase t on training	t	P
			Control	Trained			
protein	p-NPPase	4	6.456 ± 0.091 (27)	6.533 ± 0.096 (27)	1	0.5576	N.S.
		7	9.165 ± 0.159 (30)	9.783 ± 0.208 (28)	6.7	1.5802	<0.05
	β-GUase	4	0.038 ± 0.002 (27)	0.069 ± 0.002 (27)	19.0	3.2938	0.01
		7	0.102 ± 0.005 (30)	0.185 ± 0.024 (28)	81.4	3.5325	<0.001
	β-GAase	4	1.599 ± 0.038 (27)	1.709 ± 0.041 (27)	8.8	1.9794	<0.05
		7	2.401 ± 0.081 (30)	3.301 ± 0.234 (28)	57.5	3.7280	<0.001
wt-weight	p-NPPase	4	1.027 ± 0.021 (27)	1.053 ± 0.018 (27)	2.5	0.9708	N.S.
		7	1.149 ± 0.022 (30)	1.225 ± 0.018 (28)	6.6	2.6729	<0.01
	β-GUase	4	9.22 ± 0.40 (27)	11.13 ± 0.41 (27)	20.7	3.3314	<0.01
		7	11.63 ± 0.65 (30)	10.23 ± 2.79 (28)	78.1	3.1962	<0.01
	β-GAase	4	0.255 ± 0.007 (27)	0.276 ± 0.008 (27)	8.2	2.0039	<0.05
		7	0.303 ± 0.011 (30)	0.415 ± 0.028 (28)	37.0	3.7907	<0.001

the effect of physical training on the total activities of p-nitrophenylphosphatase (p-NPPase), β-glucuronidase (β-GUase) and β-N-acetylglucosaminidase (β-GAase) in crude homogenates from the skeletal muscle of 4 and 7 months old mice. The enzymatic activities are expressed as nmoles p-nitrophenol formed per mole either per protein or per mg wet-weight of muscle with the exception of β-GUase per wet-weight, here the activity is pmoles per min per mg. The figures denote mean values ± S.E. and within brackets the number of animals per group. For further experimental details: See Material and methods.

long-lasting). Furthermore, the investigation was extended to include the often omitted training.

With this experimental design data was also obtained on the activity of acid hydrolase in the skeletal muscle of mice of different ages.

Material and methods

Animals

F₁-hybrid mice of B6D2F₁/BOM-strain (C. L. Bomholtgård Ltd, Ry, Denmark) were used for the experiment. At the beginning of the experiment their body weight was 77.1 ± 0.2 g (All data is given as mean \pm S.E.) and their age 60 (range 53–67) days.

The mice were randomly divided into two main groups—either the younger group studied at the age of 30 weeks (113 ± 1 days) or the older group studied at the age of 30 weeks (208 ± 3 days). Within each group there were 3 subgroups, the first being the untrained controls (C), which were living under cage conditions with free access to commercial solid food (Hankkija, Finland) and tap water at all times. The second subgroup was the training group (TR), which ran on a motor-driven treadmill according to a programme (see below) up to the day they were killed. The third subgroup was the detrainment group (S), which terminated the training at certain intervals before being killed (see below).

In the younger group the training of the two subgroups TR and S was very intense. The training by running for 60 min at a speed of 17.6 m/min daily (7 days a week) on the motor-driven treadmill. The work load was increased to three periods of running per day (30 min at 17.6 m/min + 45 min at 17.6 m/min before noon and 75 min at 17.6 m/min in the afternoon). The training remained at this level for the TR group up to the day the mice were killed. The other subgroup (S) stopped training either 3, 7, 14 or 21 days before they were killed. During these periods they were caged in the same manner as the controls.

In the second main group—the older mice—the training of the TR and S subgroups was moderate. The training by running on the treadmill for 60 min at 20.5 m/min 5 days a week. The training remained at this level for the TR subgroup. The other subgroup stopped training 7, 21 or 42 days before the day they were killed.

During the longer training period an increase in aggressive behaviour and higher mortality were observed so that 28 of 107 mice died, mainly due to accidents on the treadmill or injuries caused by fighting with each other.

Enzyme assays

The mice were killed by dislocation of the neck, and the skin of the right leg was immediately removed. The calf cut off and chilled in an ice-cold homogenization medium (see below). The muscle tissue was moved from bone and connective tissue as carefully as possible. After weighing, the muscle was homogenized in twenty times (weight to volume) of the homogenization medium. This was a solution of 10 mM KCl, 30 mM NaHCO₃ and 6 mM EDTA, pH adjusted to 7.4 with HCl. Homogenization was performed in a Potter Elvehjem homogenizer with a teflon pestle for 3 min. After homogenization all connective tissue which could be detected was removed and weighed. The remaining homogenate was used for the enzyme activities after the addition of Triton X 100 to a final concentration of 0.1%. The homogenization procedure was performed at 0–4°C. Acid hydrolase assays were performed essentially according to Barrett (1972) in the following way:

p-Nitrophenyl phosphate (acid phosphatase, E.C. 3.1.3.2) was estimated by incubating 0.1 ml homogenate together with 1.5 ml 0.1 M acetate buffer pH 5.0, and 0.5 ml 20 mM p-nitrophenyl phosphate for 60 min at 37°C with 5 min preincubation before addition of a substrate. After incubation 5.0 ml 0.1 M NaOH was added and the extinction at 420 nm was estimated against a blank, in which NaOH was added before the substrate.

β -Glucuronidase (E.C. 3.2.1.31) was estimated by incubating 0.1 ml homogenate with 0.9 ml 0.1 M acetate buffer pH 4.2. After 5 min preincubation 0.5 ml 5 mM p-nitrophenylglucuronide was added. Incubation lasted for 18 h at 37°C. The reaction was stopped by adding 3.0 ml 0.4 M glycine buffer pH 10. After centrifugation the extinction at 420 nm was measured. A blank reaction was performed by adding glycine buffer before the homogenate.

β -N-acetylglucosaminidase (E.C. 3.2.1.30). 0.1 ml homogenate was incubated together with 0.1 M citrate buffer pH 4.8, 0.4 ml redistilled water and 0.5 ml 5 mM p-nitrophenyl-N-acetylglucosamine.

at 37°C with 5 min preincubation before addition of the substrate. After 60 min incubation 2.0 ml 10N NaOH was added and the reaction at 430 mμ was measured against a blank in which NaOH was the substrate.

Protein contents of the homogenates as determined according to the method of Lowry *et al.* (1951) using 0.5 and 2.0 ml 1 M NaOH at 40°C for 80 min and using bovine serum albumin for the standard. In order to find out if there were any differences between "tissue wet weight" and "protein content" as far as reference, the enzyme activities were calculated in both ways. The results were very similar (I-III) indicating that the treatment (training and detraining) did not induce edema or any similar effect.

Results are statistically compared by means of Student's *t*-test. All substrates were obtained from Chemical Co.

Results

of age

Activity of the acid hydrolases was greater in the skeletal muscle of the older control than in that of the younger controls. The sizes of differences were not equal for the three enzymes but greatest in β -glucuronidase and lowest in p-nitrophenylphosphatase activity (Table I).

of training

Skeletal exercise induced increased activities of the three acid hydrolases in the skeletal muscle of mice except p-nitrophenylphosphatase in the younger intensively trained animals (Table II). Thus β -glucuronidase activity increased approx. 80% in the moderately trained, and 20% in the intensively trained younger mice. β -N-acetylglucosaminidase activity increased 37% and 7%, respectively and p-nitrophenylphosphatase activity increased approx. 7% in the moderately trained group.

of terminated training

p-nitrophenylphosphatase was unaffected by detraining, although its activity resembled that of the trained animals (Table III, Fig. 1). The activity of β -glucuronidase was distinctly lower after training had been stopped for 7 and 21 days, respectively in the moderately trained group. After 42 days of detraining the activity of β -glucuronidase in the 3 group increased and no significant difference was observed between it and either control or the trained groups. In the younger intensively trained groups the activity of glucuronidase was higher after 3 days of detraining than in the trained mice or in the controls. After 7 and 14 days the activity was at the same level as in the trained animals (Table III, Fig. 1).

The activity of β -N-acetylglucosaminidase followed a similar pattern to that of β -glucuronidase in the younger, intensively trained animals, although the differences after 7 and 14 days were not statistically significant. In the muscle of older moderately trained animals, this enzyme had a higher activity than in the corresponding controls 7 days after training had terminated. Fourteen days later the activity had lessened further and was between the two other groups. Forty-two days after training had ceased the activity of β -N-acetylglucosaminidase had increased and was close to that of the trained animals (Table III, Fig. 1).

The duration of training and terminated training lasted for different periods in the younger and older animals and only a period of 7 days detraining occurred in both age groups.

TABLE III A Effect of terminated training of different duration on the activities of acid ischry skeletal muscle of old and young mice (per protein content).

Enzyme	Age (months)	Group	Activity at the end of		
			1st period	2nd period	3rd period
p-NPP-ase	4	S	6.67 ± 0.14 (9)	6.30 ± 0.16 (9)	6.87 ± 0.24
		C	6.43 ± 0.14 (9)	6.43 ± 0.18 (9)	6.88 ± 0.17
		TR	6.51 ± 0.12 (9)	6.39 ± 0.16 (9)	6.89 ± 0.25
	7	S	10.30 ± 0.29 (10)	8.85 ± 0.17 (10)	10.17 ± 0.34
		C	9.76 ± 0.20 (10)	8.43 ± 0.23 (10)	9.38 ± 0.25
		TR	10.37 ± 0.36 (10)	8.75 ± 0.24 (10)	10.21 ± 0.38
β-GU-ase	4	S	0.070 ± 0.004 (9)	0.069 ± 0.004 (9)	0.075 ± 0.004
		C	0.053 ± 0.003 (9)	0.057 ± 0.004 (9)	0.064 ± 0.004
		TR	0.061 ± 0.003 (9)	0.070 ± 0.004 (9)	0.076 ± 0.004
	7	S	0.113 ± 0.006 (10)	0.101 ± 0.007 (10)	0.124 ± 0.015
		C	0.103 ± 0.013 (10)	0.093 ± 0.005 (10)	0.107 ± 0.007
		TR	0.184 ± 0.036 (10)	0.163 ± 0.024 (10)	0.214 ± 0.040
β-GA-ase	4	S	1.711 ± 0.053 (9)	1.741 ± 0.072 (9)	1.775 ± 0.082
		C	1.533 ± 0.049 (9)	1.620 ± 0.068 (9)	1.644 ± 0.073
		TR	1.563 ± 0.038 (9)	1.764 ± 0.058 (9)	1.801 ± 0.084
	7	S	2.799 ± 0.146 (10)	2.517 ± 0.109 (10)	2.789 ± 0.165
		C	2.351 ± 0.104 (10)	2.461 ± 0.083 (10)	2.591 ± 0.096
		TR	3.095 ± 0.293 (10)	3.108 ± 0.234 (10)	3.802 ± 0.638

TABLE III B Effect of terminated training of different duration on the activities of acid ischry skeletal muscle of older and younger mice (per muscle wet-weight).

Enzyme	Age (months)	Group	Activity at the end of		
			1st period	2nd period	3rd period
p-NPP-ase	4	S	1.031 ± 0.022 (9)	1.023 ± 0.026 (9)	1.097 ± 0.051
		C	0.982 ± 0.040 (9)	1.039 ± 0.036 (9)	1.080 ± 0.053
		TR	1.021 ± 0.013 (9)	1.015 ± 0.033 (9)	1.124 ± 0.065
	7	S	1.137 ± 0.039 (10)	1.16 ± 0.028 (10)	1.281 ± 0.037
		C	1.104 ± 0.030 (10)	1.142 ± 0.037 (10)	1.221 ± 0.037
		TR	1.191 ± 0.036 (10)	1.209 ± 0.020 (10)	1.389 ± 0.078
β-GU-ase	4	S	10.84 ± 0.48 (9)	11.11 ± 0.46 (9)	12.46 ± 0.82
		C	8.17 ± 0.58 (9)	9.18 ± 0.36 (9)	10.30 ± 0.88
		TR	9.66 ± 0.60 (9)	11.01 ± 0.66 (9)	12.72 ± 0.54
	7	S	12.19 ± 0.55 (10)	9.05 ± 0.48 (10)	21.54 ± 5.34
		C	11.48 ± 1.18 (10)	8.54 ± 0.41 (10)	14.04 ± 0.96
		TR	1.07 ± 4.03 (10)	14.49 ± 1.90 (10)	26.35 ± 7.85
β-GA-ase	4	S	0.263 ± 0.010 (9)	0.282 ± 0.010 (9)	0.292 ± 0.012
		C	0.234 ± 0.012 (9)	0.261 ± 0.012 (9)	0.269 ± 0.013
		TR	0.245 ± 0.007 (9)	0.280 ± 0.012 (9)	0.303 ± 0.014
	7	S	0.307 ± 0.015 (10)	0.334 ± 0.014 (10)	0.466 ± 0.029
		C	0.259 ± 0.018 (10)	0.309 ± 0.014 (10)	0.341 ± 0.017
		TR	0.358 ± 0.036 (10)	0.422 ± 0.033 (10)	0.473 ± 0.076

The effect of terminated training on the total activities of acid p-nitrophenylphosphatase (p-NPP-ase), β-glucuronidase (β-GU-ase) and β-N-acetylglucosaminidase (β-GA-ase) in crude homogenates of skeletal muscle from 4 and 7 months old mice. The enzymatic activities are expressed as nmoles p-nitrophenol formed per min per mg protein (Table III A) or per mg muscle wet-weight (Table III B). β-GU-ase in Table III B is expressed as pmoles per min per mg muscle wet-weight.

1st, 2nd and 3rd periods denote in the 4 months group 3, 7 and 14 days after training had been terminated. The corresponding periods in the 7 months group are 7, 21 and 42 days. The group abbreviations are: terminated trained (S-stopped), controls (C) and training (TR) mice. and denote statistical significant limits $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively when compared.

hypothesis that aging is genetically governed has been proposed by Goldstein (1971), if it is true, might be the explanation for the high activity of the acid hydrolases.

Effect of training

Training increases the activity of acid hydrolases in skeletal muscle and the increase is more pronounced in older mice than in young (Table II). Although the two groups were subjected to different training programmes we are of the opinion that age has a greater influence on the increase than the intensity of training. The reason for this is that, in an earlier publication (Viikko *et al.* 1974) with young mice trained in a similar way as the older mice in this study the increases in the activity of β -glucuronidase was equal to that found in the older group, which had trained more intensively. It should also be noticed that in the group of older trained mice there were a few animals showing extremely high activities of β -glucuronidase (and to lesser extent of β -N-acetylglucosaminidase) which increased the mean of the data (Table II).

Viikko and Bird (1970) have obtained results which suggest that the activity of β -glucuronidase is partly derived from macrophages and connective tissue, while the activity of acid phosphatase (Le. p-nitrophenylphosphatase) is mostly derived from the muscle cells. A bimodal distribution might be the explanation for the small or non-existing effect of training on acid phosphatase activity in contrast to the marked effects on β -glucuronidase and β -N-acetylglucosaminidase (Table II and III). However recent histochemical studies (Gutman and Gutman 1976) show the activity of all these three acid hydrolases also in muscle. Thus the different effect of training on the activity of acid phosphatase and the two other enzymes cannot simply result only in changes in cell populations and remains so far unexplained.

Effect of terminated training

Koer *et al.* (1972) report a loss of muscle fibres in guinea-pigs during detraining and Reisman and Reitman (1977) report a reduction in the activity of succinate dehydrogenase and cytochrome oxidase in humans during detraining. During such a reduction of activity of cell components an increase of the activity of acid hydrolases would be expected. Only after 42 days of terminated training induced in the skeletal muscle of younger mice an activity of β -glucuronidase and β -N-acetylglucosaminidase which exceeded that in the younger mice under training (Table III, Fig. 1). This fits our original hypothesis. In all other groups the activities were not higher after termination of training than during training.

Even after 42 days of terminated training gave different results for the two age groups. Thus in the older animals the activity of the acid hydrolases was similar in the control group and in the trained group but in the younger mice these levels were similar as between the detrained and trained mice. The activity of the older detraining group remained low for at least 21 days after training had stopped (Table III, Fig. 1). Activities of enzymes of energy metabolism decrease after 5 weeks detraining in horses of respective age as our older mice but again after 10 weeks detraining a consistent but inexplicable increase in enzyme activities was observed (Guy and Snow 1977).

It is possible that age causes a lag phase in the functions of catabolic mechanisms after

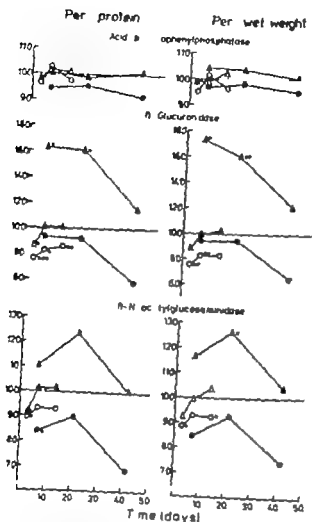


Fig. 1 The relative activities (fetal acid p -nitrophenylphosphatase, β -glucuronidase and β -N-acetylglucosaminidase) of control (circles) and training mice (squares) compared to terminated training (100) during different periods after training in mice. Open symbols denote control mice and filled symbols denote training mice. 20 ± 3 days. Statistically significant differences from the values for the terminated groups are indicated by * corresponding to $p < 0.05$, $p < 0.01$, $p < 0.001$ respectively. For further experimental details see Material and methods.

On this occasion, however, the activities of the acid hydrolases were closer to those of the trained group in the younger animals and more similar to those of the controls in the older group (Fig. 1).

Age difference

The oxidative capacity of skeletal muscle fibre decreases with aging in guinea-pigs as has been shown in histochemical studies (Faulkner *et al.* 1972, 1973; Maxwell *et al.* 1973). The activity of acid phosphatase in different tissues of old mice—including skeletal muscle—is higher than in young animals (Youhousky-Goro and Pathmanathan 1968). This is also true for β -glucuronidase and β -N-acetylglucosaminidase in skeletal muscle (Table I). Furthermore, the magnitude of the increase in activity of the acid hydrolases induced by training is greater in the older mice (Table II).

The difference caused by age in the oxidative capacity of skeletal muscle can to a large extent be explained by the observed shift of the fibre composition in guinea-pigs (Faulkner *et al.* 1972, 1973; Maxwell *et al.* 1973). Whether this is also true for the acid hydrolases remains to be studied.

Discussion

Effects on fluid balance induced by non-febrile intracerebroventricular infusions of PGE_2 , $\text{PGF}_{2\alpha}$ and arachidonic acid in the goat

By

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Abstract

LEXELL, L. G. *Effects on fluid balance induced by non-febrile intracerebroventricular infusions of PGE_2 , $\text{PGF}_{2\alpha}$ and arachidonic acid in the goat* Acta physiol. scand. 1978. 104. 225-231

Arachidonic (PG) E_2 , $\text{F}_{2\alpha}$ (30 $\mu\text{g}/\text{kg}\cdot\text{min}^{-1}$) and arachidonic acid (150 and 300 $\mu\text{g}/\text{kg}\cdot\text{min}^{-1}$) are infused for 30 min into the lateral cerebral ventricle of conscious hydrated and non-hydrated goats. Like previously shown in conscious PGE_2 , PGE_2 is found to inhibit the thirst diuresis and cause some increase in renal sodium excretion in the hydrated animal, and to elicit thirst in the non-hydrated goat. The effect of PGE_2 was enhanced when hypertonic (0.25 M) NaCl was simultaneously infused into the ventricle. The antidiuretic effect of $\text{PGF}_{2\alpha}$ was less pronounced, and drinking only occasionally occurred. When PG was infused into the non-hydrated animal only a small, post-infusion reduction of the thirst was observed. When arachidonic acid was administered into the hydrated goat neither the PGE_2 , nor arachidonic acid affected the temperature regulation of the animals. The possibility is discussed that lack of a lytic response as due to the choice of plasma-cryoprecipitate as material for the cerebral infusions. It is suggested that PGE_2 might have participated with CSF Na^+ in stimulating juxta-ventricular plots involved in the control of fluid balance. The experiments do not support the concept that PGE_2 is a serum constituent. Cerebral haemostasis but is pyrogen-induced fever.

Prostaglandins (PG) of different types have been identified in various parts of the brain, among them the hypothalamus (Holmes and Horton 1968). It has been suggested that these compounds may be involved in several hypothalamic functions, such as regulation of food intake, anterior pituitary secretion and body temperature (cf Wolfe 1975). That intra-cerebroventricular (IVT) or intrahypothalamic injections of PG of the E series produce an elevation of the body temperature has been demonstrated in several mammalian species, e.g. the rat and rabbit (Milton and Wendlandt 1971) and the sheep (Bligh and Milton 1973). In support of the idea that hypothalamic PG-synthesis is involved in the fever mechanism is also the observation that IVT injections of the PG-precursor arachidonic acid reduces fever in the rat (Splaninksi et al 1974).

That the hypothalamic regulation of antidiuretic hormone (ADH) secretion may be

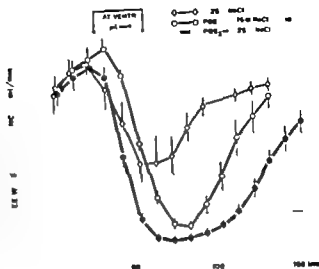
terminated training, while in young animals the destruction in the muscle, including capillairs and connective tissue, starts immediately. A similar increase is not found in animals until 42 days after training has ceased.

This study was supported by grants (9290/79/73 and 11675/79/74) from the Ministry of Education and a grant from the Ellen and Artturi Nyyssönen Foundation. Mrs Arja Mäntylä and Jussi technical assistance which is gratefully acknowledged.

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A comparison between the renal responses to IVT doses of hypertonic (0.25 M) and PGE_2 as isotonic or tonic NaCl. The experiments performed in 4 hydrated goats. Note the accentuation of response with PGE_2 and tonic NaCl are administered. Dose of 30 $\mu\text{g kg}^{-1} \text{min}^{-1}$. Vertical S.E.



Results

1 Fluid balance

Effects obtained by the IVT infusion of PGE_2 were largely identical to those previously observed in response to the same dosage of PGE_2 in the goat (Lekseil 1976). As shown in Fig. 1, the IVT infusion of PGE_2 dissolved in isotonic (0.15 M) NaCl ($n=10$) caused a marked inhibition of the water diuresis in hydrated animals. It implies that PGE_2 acted as an efficient stimulus of ADH-release. This antidiuretic effect became much accentuated when the NaCl concentration of the CSF was simultaneously elevated. The infusion of PGE_2 in 0.25 M NaCl ($n=11$) completely inhibited the water diuresis (—negative renal flow) for about 1 h, although the 0.25 M NaCl *per se* was not sufficient to induce negative flow (Fig. 1). A similar PGE_2/Na interaction was seen as regards the renal sodium excretion in the hydrated animals (Fig. 2). The IVT infusions of 0.25 M NaCl and of PGE_2 in isotonic NaCl caused only minor increases in the sodium excretion, whereas a pronounced triphasic response developed in response to the combined infusion of PGE_2 and hypertonic NaCl. In the non-hydrated goat the IVT infusion of PGE_2 in isotonic NaCl ($n=5$) invariably induced drinking. The average latency time for the dipsogenic response was 9 min and 0–140 ml of water was consumed.

$PGF_{2\alpha}$

The antidiuretic effect of $PGF_{2\alpha}$ was much less pronounced than that induced here by PGE_2 and previously by PGE_2 (Lekseil 1976). As shown in Fig. 3 the water diuresis gradually came reduced during the IVT infusion of $PGF_{2\alpha}$ in isotonic NaCl ($n=8$) into the hydrated goat. However the infusions did not induce negative renal C_{H_2O} , indicating that only small amounts of ADH were released. These infusions had no appreciable effect upon the renal sodium excretion.

affected by PG's of the E series was originally suggested by Vilhardt and Hedqvist who found that Intracarotid Infusions of PGE inhibited the water diuresis of hydrated goats. Accordingly in a later study (Leksell 1976) it was observed that IVT infusions of PGE affected various aspects of the cerebral control of fluid balance. The substance was found to stimulate the release of ADH in hydrated goats, to elicit drinking in non-hydrated goats and to interact with cerebrospinal fluid (CSF) Na⁺ in augmenting renal sodium excretion and elevating the arterial blood pressure. Unexpectedly PGE did not induce fever in the goat in spite of the fact that the IVT infusions by all probability raised the PG-concentration of the CSF way above pathophysiologically feasible levels. It has been suggested, here that pyrogen-induced fever is mediated by hypothalamic PGE₂ synthesis in the cat (Fitz and Gupta 1973), and IVT administration of PGF₂ has been observed to cause fever in the sheep (Hales *et al.* 1973). Therefore, it was of interest to extend the previous study on the goat to involve the latter two PG's and arachidonic acid.

Methods

Animals. Four adult female goats (b.wt. 31–36 kg) were used. The animals were kept in metabolism cages where all experiments were conducted and where the goats had free access to hay and water. They were maintained in positive sodium balance by receiving 6 g of NaCl added to a daily ration of coarse grain mix.

IVT implantations and infusions. The goats had a special cannula system (Åkerman, Andersson-Olsson 1973) implanted under general anaesthesia (nembutal) into one lateral ventricle about 14 d before the experimental series were started. The cannula-material was platinum-iridium in order to minimize body reaction. In all experiments CSF was observed to drain out of the cannula on gentle compression of the neck immediately before and after the infusions. Thus, free mixing of the infused material with CSF occurred in all experiments. The infusion rate was 10 µl/min and the duration 30 min. The minimum time interval between each experiment was two days.

Hydration. Hydration was accomplished by giving 100 ml/kg of 38°C water by stomach tube in the rumen 90 min before the IVT infusion was started.

Urine sampling and analyses. Urine was collected in 10 min samples via a Foley catheter. Urine flow was determined by use of an "IL" 343 flame photometer and an Adv Instruments Inc. osmometer used to measure urine and blood plasma osmolality. A plasma osmolality around 290 mosm/kg was obtained during hydration, and this value was invariably used for calculations of renal free water clearance (C_{osm}) during hydration experiments.

Solutions used for the infusions. PGE₂ (crystalline) and PGF_{2α}-tromethamine salt were generously supplied by The Upjohn Company. The PGE₂ was dissolved in 99% ethanol and was stored in samples of 1 mg (+20 µl of ethanol) at -20°C. Within one h before the infusion, 1 ml of 1.9 mmol Na₂CO₃ was added to the sample of PGE₂. The PGF_{2α}-tromethamine salt was dissolved in 0.15 M NaCl within 30 min before the experiment. Arachidonic acid (Nu Chek Prep. Inc.) was obtained from the Dept. of Chemistry, University of Illinois. It was dissolved in 99% ethanol and was stored at -20°C in a concentration of 20 mg/ml. About 15 min before the infusions were started the dissolved substances were mixed with NaCl solution of adequate volume and concentration to perform the following IVT infusions:

- 1) PGE₂ (30 ng/kg min⁻¹) in 0.15 M NaCl
- 2) PGE₂ (30 ng/kg min⁻¹) in 0.25 M NaCl
- 3) PGF_{2α} (30 ng/kg min⁻¹) in 0.15 M NaCl
- 4) Arachidonic acid (150 ng/kg min⁻¹) in 0.15 M NaCl
- 5) Arachidonic acid (300 ng/kg min⁻¹) in 0.15 M NaCl

For comparison infusions of simply 0.25 M NaCl were also made. Three control infusions combined ethanol/0.15 M NaCl (in the same proportions as in the PGE₂ and arachidonic acid experiments) were made in the hydrated animal.

2. Temperature regulation

of the experiments (PGE_2 $n=10$, PGF $n=5$ and arachidonic acid $n=7$) the rectal temperature was measured 10 min before and after the infusions. Mean pre-infusion and infusion values were 39.1 ± 0.0 and $39.0 \pm 0.1^\circ\text{C}$ respectively. The maximum temperature rise observed after the infusions was 0.3°C (up- or downwards). Piloerection, shivering or febrile reactions were not observed in any of the present expts.

Discussion

It appears to deserve particular attention in the present study is that neither IVT infusions of the tested PGs, nor of arachidonic acid influenced the body temperature of the goat. The hypothesis that prostaglandins are mediators of the febrile response to pyrogens acts within the preoptic/anterior hypothalamic region is supported from several studies. Among the supports are the observations in almost all mammalian species tested that PGs of the E series induce fever when administered either into the cerebral cortex, or directly into brain tissue near the frontal border of the third ventricle (cf. Hales 1974). Only two exceptions have been reported, the echidna and the goat. Injections of PGE_1 and E_2 into the lateral ventricle of echidnas were found ineffective in raising the temperature (Baird, Hales and Lang 1974), and infusions of PGE into the third ventricle (Hales and Leisell 1975) and lateral ventricles (Leisell 1976) were not observed to cause fever or any obvious activation of heat conserving mechanisms in the goat. The results reported here do not provide any support for the possibility that cerebral synthesis of some

PG other than PGE_2 would instead be the mediator of pyrogen fever in the goat. Of interest for comparison are similar studies performed in a closely related ruminant species, the sheep. These investigations agree that PGE as well as PGE_2 have a febrile action when infused into the lateral ventricle of the adult animal (Bligh and Milton 1973; Hales *et al.* 1973). However Pittman, Veale and Cooper (1975) have reported that such injections often do not cause fever in the newborn lamb. Furthermore, the same authors recently (1977) did not succeed in producing fever by preoptic/anterior hypothalamic microinjections of PGE_1 or PGE_2 in the adult sheep, although bacterial pyrogen often had this effect when infused at the same cerebral sites. Nonetheless, in that study it was confirmed that IVT infusions of larger amounts of PGE induce fever. The conclusions reached by Pittman *et al.* (1977) were that the sheep may be able to develop fever without the central involvement of PG, or if PG does play a role, it may act within the brain at a site other than the preoptic/anterior hypothalamic region.

The previous goat and some of the sheep studies referred to above, together with the present results provide substantial evidence against the concept that PG has a general importance as an intermediary in the fever mechanism. However it remains an intricate problem why the IVT administration of various PGs and arachidonic acid did not induce fever in the goat, whereas this has been a rather consistent effect in other investigated adult mammals. It could hardly be a question of dosages since the amount of PG infused into the goat (approximately $30 \mu\text{g}$ in 30 min) was of the same order as that found to cause a considerable elevation of body temperature in the sheep (Hales *et al.* 1973). Although a

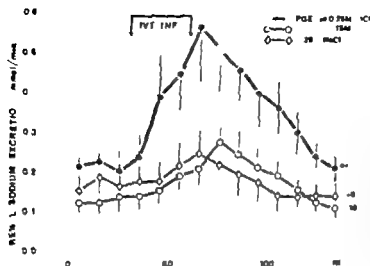


Fig. 2. Pronounced antidiuretic response in the hydrated goat to IVT infusion of PGE_2 at 1 tonic (0.25 M) NaCl (dotted comparison) is shown the weaker effects of PGE_2 in isotonic saline and of solely the hyper-NaCl. Dosage of PGE_2 0.1 mg/ml . No of animals 4. Vertical bars = S.E.

In only 2 out of 5 expts. performed in the non-hydrated goat did the animals drink during the infusion of $\text{PGF}_{2\alpha}$ dissolved in isotonic saline. Drinking occurred after 6 and 25 min the amounts of water drunk were 700 and 140 ml respectively.

Arachidonic acid

No or little reduction of the water diuresis occurred during the IVT infusions of arachidonic acid in the hydrated goat, regardless of whether the large ($n=6$) or the small dose ($n=3$) was administered. However a temporary post infusion drop in the renal $\text{C}_{\text{H}_2\text{O}}$ was observed which was most pronounced after that the large amount of the acid had been infused (Fig. 3). No obvious effect on the renal sodium excretion was observed in these expts.

Control infusions

The control infusions of solvent performed in hydrated animals (see methods) caused no reduction in renal $\text{C}_{\text{H}_2\text{O}}$ or change in the renal sodium excretion.

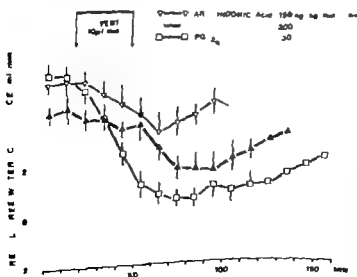


Fig. 3. Influence of IVT infusion of arachidonic acid and $\text{PGF}_{2\alpha}$ (in 0.15 M NaCl) on the control of the water diuresis in 4 hydrated goats. Note that $\text{PGF}_{2\alpha}$ has a considerably weaker antidiuretic effect than PGE_2 (see Fig. 1). Vertical bars = S.E.

Temperature regulation

In the experiments (PGE_2 $n=10$, $\text{PGF}_{2\alpha}$ $n=5$, and arachidonic acid $n=7$) the rectal temperature was measured 10 min before and after the infusions. Mean pre-infusion and infusion values were 39.1 ± 0.0 and $39.0 \pm 0.1^\circ\text{C}$ respectively. The maximum temperature observed after the infusions was 0.3°C (up- or downwards). Piloerection, shivering or febrile reactions were not observed in any of the present expts.

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The previous goat and some of the sheep studies referred to above, together with the present results provide substantial evidence against the concept that PG has a general importance as an intermediary in the fever mechanism. However, it remains an intricate problem why the IVT administration of various PGs and arachidonic acid did not induce fever in the goat, whereas this has been a rather consistent effect in other investigated adult mammals. It could hardly be a question of dosages since the amount of PG infused in the goat (approximately $30 \mu\text{g}$ in 30 min) was of the same order as that found to cause a considerable elevation of body temperature in e.g. the sheep (Hales *et al.* 1973). Although a

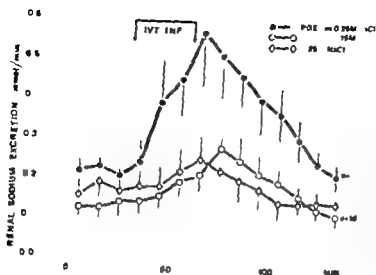


Fig. 2. Proximal sodium response in the hydrated goat - IVT infusion of PGE_2 in isotonic (0.25 M) NaCl (for comparison are shown the weaker effects of PGE_2 in saline and of solely the hypotonic NaCl. Dose of PGE_2 9.1 μg kg^{-1} min^{-1} . No of animals 4. Vertical bars = S.E.

In only 2 out of 5 expts. performed in the non-hydrated goat did the animals drink during the infusion of PGF_2 dissolved in isotonic saline. Drinking occurred after 6 and 26 min; the amounts of water drunk were 700 and 140 ml respectively.

Arachidonic acid

No or little reduction of the water diuresis occurred during the IVT infusions of arachidonic acid in the hydrated goat, regardless of whether the large ($n=6$) or the small dose ($n=4$) was administered. However, a temporary post infusion drop in the renal $\text{C}_{\text{H}_2\text{O}}$ was observed which was most pronounced after that the large amount of the acid had been infused (Fig. 1). No obvious effect on the renal sodium excretion was observed in these expts.

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The control infusions of solvent performed in hydrated animals (see methods) caused no reduction in renal $\text{C}_{\text{H}_2\text{O}}$ or change in the renal sodium excretion.

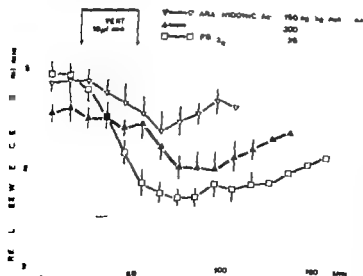


Fig. 3. Influence of IVT infusion of arachidonic acid and PGE_2 (in 0.15 M NaCl) on the control water diuresis in 4 hydrated goats. Note that PGE_2 has a considerably weaker antidiuretic effect than PGE_2 (see Fig. 1). Vertical bars = S.E.

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species difference can not be excluded as the explanation, it appears more likely that a particular experimental condition was the reason for the unresponsiveness of the goats. In the present and previous PG experiments performed in this species, the permanent cannulae were made of a material causing very little foreign body reaction (gold-iridium), whereas stainless steel cannulae (more likely to cause deposits of metallic salts) have been used in corresponding experiments performed in other mammals. The unique material might have been the ultimate reason why the goats did not respond well to the IVT infusions of PGs and the PG precursor. If so, it implies that the fever due to IVT infusions in other species might have been secondary to PG-induced liberation of endogenous pyrogen from local inflammatory processes elicited as a foreign body reaction to the cerebral implantations.

Juxtaventricular sodium sensitive receptors, the activation of which may be dependent upon Na⁺ K ATPase activity apparently play an important role in the cerebral control of fluid balance (cf Andersson 1977). Earlier (Leksell 1976) it was found that the IVT administration of PGE₁ like elevated CSF [Na⁺], stimulates thirst, ADH release, and sodium excretion. It led to the suggestion that PGE₁ might affect periventricular Na⁺ transport in a manner which stimulates receptors which primarily are sodium sensitive. Direct support for that idea was obtained by the previous observation that PGE₁ elevated Na⁺ K ATPase activity (Limas and Cohn 1974). Also PGE₂ was then found to stimulate Na⁺-transporting enzyme, and was in the present study observed to affect the fluid balance of the goats in the identical manner as PGE₁. It appears possible, therefore, that PGE₁ and E₂ have the same action on juxtaventricular receptors involved in the control of fluid balance. The present experiments indicate that PGF₁ and arachidonic acid are much less efficient than PGE₁ and E₂ in that respect. However the mere fact that IVT administration of PGE₁ and E₂ are relatively efficient stimuli of thirst and ADH secretion can by no means be regarded as evidence that cerebral PG synthesis might play a physiological role in the control of fluid balance. The amounts of the substances needed to elicit these effects were obviously pharmacological. A CSF concentration of PG in the order of 1 ng/ml has been found in laboratory animals (cf Hellon 1974). That concentration level must have been considerably exceeded during the IVT infusions of PG performed in the goat.

This work was supported by the Swedish Medical Research Council (Project 04X-00503) and by grant from Karolinska Institutet. I am indebted to Dr Jan Svensson for invaluable discussions. The gift of PGE₁ and PGF₁ from The Upjohn Company is gratefully acknowledged.

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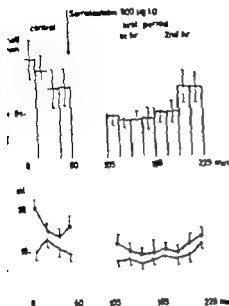


Fig. 1

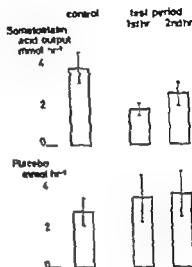


Fig. 2

1. Basal acid secretion (mmol/15 min) before and after intragastric administration of 300 µg somatostatin. Mean \pm S.E. n=6. $p < 0.05$.

2. Basal gastric acid output (mmol hr⁻¹) during control hour and following intragastric administration of somatostatin (S-6) or placebo (P-6). Mean \pm S.E. $p < 0.02$.

4. 0.63 mmol ($p < 0.02$) and 1.26 ± 0.43 mmol ($p < 0.02$) and the acid output was respectively 49.5 ± 9.6 and 69.6 ± 10.9 percent of that in control hour.

As a comparison (Fig. 2) placebo instillation did not change significantly the basal acid secretion which during the two test hours was 132.8 ± 28.6 and 135.1 ± 14.8 percent of that control hour.

1. 300 µg intragastric somatostatin did not significantly change the gastric acid response to histamine, the average acid output in 6 healthy subjects was 91.8 ± 6.8 and 106.9 ± 21.2 percent of control values during the first and second hour of stimulation.

Discussion. Somatostatin, first isolated from the hypothalamus was soon detected in the cells localized in the pancreas, stomach and in a decreasing number along the small intestine (Hökfelt *et al.* 1975, Polak *et al.* 1975). Somatostatin is a potent inhibitor of several secretory and motility functions in the gastrointestinal tract (Bloom *et al.* 1974, Bloom *et al.* 1975, Larssén *et al.* 1975, Johansson *et al.* 1978). These inhibitory effects might be due to blocking of the gastrointestinal hormone release (Boden *et al.* 1975, Bloom *et al.* 1975, Chappel *et al.* 1977), but the gastric antisecretory effect is at least partly due to direct action on the parietal secretory cell (Barrios *et al.* 1975).

2. Vagal stimulation releases somatostatin into the antral lumen of cats during acid perfusion, with concomitant depression of the gastric release (Uvnäs-Wallensten *et al.* 1977).

Effects of intragastrically administered somatostatin on basal and pentagastrin stimulated gastric acid secretion in man

By

C. JONANSSON, O. WISÉN, H. KOLLBERG, K. UVNÄS-WALLÉN and S. EITZKE

Given intravenously somatostatin is a potent inhibitor of gastric acid secretion, in a being directed towards both the gastrin producing G-cell (Bloom *et al* 1974) and the parietal cell (Barros *et al* 1975). The physiological importance of somatostatin in the regulation of gastric acid secretion is unclear. A role as a paracrine regulator of the pH-dependent release of histamine has been postulated (Uvnäs-Wallén *et al* 1977). If somatostatin acts locally on the G-cell then intragastrically administered exogenous somatostatin might inhibit gastric acid secretion. This was tested by examining the effect of intragastric somatostatin on basal and pentagastrin stimulated gastric acid secretion in healthy human subjects.

Method. Basal gastric secretion was collected in 9 healthy male subjects aged 21 to 29, mean 25 years, who were intubated after an overnight fast (Ryle tube 14 FG Portex). The position of the tube in the stomach was controlled by fluoroscopy and the gastric residual removed. With the subject lying on his left side and the saliva being withdrawn by a suction device, basal gastric secretion was collected by siphonage and gentle manual suction during 15 min control periods. Thereafter was injected 9 µg cyclic somatostatin (KABI, Sweden) dissolved in 4 ml 0.15 M NaCl. After washout of the tube with 10 ml saline and 10 ml air the subject turned round during 15 min, spending an equal period of time on each side of the body and was then allowed to walk around. At 42 min after the instillation, the gastric residual was again removed, the subject lay down on his left side and gastric juice was collected in eight 15 min periods. One experiment was rejected, due to bile standing of more than two collections during the period and results from 8 expts. reported.

Placebo expts. with gastric intubation of 0.15 M NaCl performed in 6 subjects aged 22 to 28, mean 25 years. The effect of somatostatin on pentagastrin stimulated gastric acid secretion was examined in 6 healthy male subjects aged 22 to 28, mean 24 years, who served as their own controls. The order of the expts. was randomised. The general procedure was as described above. After complete aspiration of the stomach 15 µg cyclic somatostatin, or in controls, isotonic saline, was instilled through the gastric tube. An 1 ml solution of pentagastrin, 0.6 µg kg⁻¹ B (Peptal Ion ICT) was started 45 min thereafter at a rate of 1 ml h⁻¹. The tube was removed and gastric secretion collected in 15 min periods.

Volumes of the gastric secretion were measured and the acidity of gastric samples was determined by automatic titration with 0.1 M NaOH to pH 7 (Radiometer Copenhagen). Means are given \pm S.E. The paired Student's *t*-test was used to test significance. In each individual test of the basal secretion the output during the control hour was compared with the first and second test hour. Likewise was the acid output during the control hour compared with each 15 min collection period during the test period.

The study was approved by the Ethical Committee at Karolinska Hospital and informed consent was obtained from each subject.

Results. Instillation of 500 µg somatostatin into the stomach of healthy subjects reduced significantly the basal acid secretion by depressing both volumes and acidity of the secreted gastric juice (Fig. 1). The reduction during the first and second test hour was on average

Changes in muscle fibre type distribution in man after physical training

A sign of fibre type transformation.

By

EVA JANSSON, BERTIL SÄÖBOM AND PER TÖRCH

Experiments with cross-innervation and electrical stimulation of skeletal muscle in animal (Birley and Close 1971, Pette and Schmez 1977) and man (Miyasat *et al* 1976) indicate possibility of transforming type I (slow twitch) to type II (fast twitch) muscle fibres and *vice versa*. Changes in the percentages of either fibre type related to physical training have not been reported in man. However endurance training decreased the type II B/II A ratio whereas immobilization increased this ratio (Andersen and Henriksen 1977, Jansson and Sjöer 1977, Häggmark 1978). These observations indicate a reversible transformation between type II A and II B fibres. A recent investigation on endurance trained subjects has revealed a high percentage of type II C fibres in the trained muscles (Jansson and Kaijser 1977). These normally rare fibres have been assumed to be undifferentiated or intermediate fibres involved in reinnervation or motor unit transformation (Morris 1970, Kugelberg 1976). The significance of the elevated occurrence of type II C fibres in the well-trained subjects mentioned above is difficult to evaluate but might be an indication of a progressing fibre type transformation process. The aim of the present study was to throw further light on questions concerning fibre type transformation by studying long-term effects of different types of physical training on the percentages of different fibre types.

Four long-distance runners participated in the study. Mean values of age, weight and height were 21 (17-28) yrs, 67 (61-70) kg and 179 (174-187) cm respectively. Maximal oxygen intake averaged 74 (71-77) ml kg⁻¹ min⁻¹. Their training activity could essentially be divided into two periods. 1. "Aerobic" endurance training (18 (13-20) weeks), i.e. long-distance running with an intensity corresponding to the "aerobic-anaerobic threshold level" as defined by Mader *et al* (1976) (70-80% of VO₂ max.). The cumulated distance per week was 110 (78-160) km. 2. "Anaerobic" training (11 (7-13) weeks) was mainly performed as interval training 2-3 times a week on track at 90-100% of their VO₂ max. This training resulted in high blood lactate concentration 14.0 (11.6-15.4) mmol/l. The cumulated distance per week, which also included distance running, was 71 (40-118) km per week. Needle biopsies were taken from vastus lateralis after both training periods. For subjects 1 and 2 the first biopsy was taken after the "aerobic" and the second after the "anaerobic" training whereas the order was reversed for subjects 3 and 4. Serial sections of the biopsies were stained

Based on this finding and the close vicinity of the somatostatin producing D-cell and G-cell in the antral mucosa a paracrine action has been postulated, implying that somatostatin may regulate the gastrin release by local action on the G-cell (Uynäs-Wallensten *et al.*).

If somatostatin exerts actions during and after its release to the gastric lumen, its actions should expectedly be reproduced by intragastrically instilled exogenous somatostatin. The reduction of the basal gastric acid secretion recorded in this study after intragastric administration of somatostatin supports this possibility. That this inhibition is a local effect on the antral G-cell has, however, to be confirmed by determination of gastrin levels after intragastric somatostatin. It should also be considered that desat effects might be mediated by small amounts of somatostatin absorbed into the enteric mucosa since i.v. doses less than 30 µg/h are followed by a significant reduction of the gastric secretion (Johansson 1978, unpublished data).

In contrast to the inhibition of the basal gastric acid secretion, intragastric somatostatin did not inhibit the pentagastrin stimulated secretion, indicating that the peptide when administered had no direct effect on the stimulated parietal cell. The present data are, however, inadequate for the exclusion of such a possibility which has to be further tested with higher somatostatin doses instilled into the fundic part of the stomach.

The gastric antisecretory effects of intragastrically administered somatostatin have important clinical implications provided that the hormone, when given by this route, is not metabolized at concentrations known to suppress the release of a series of different hormones like histamine, glucagon, growth hormone and other. Such a broad spectrum of effects of intragastrically given somatostatin is the principle obstacle for its introduction into clinical medicine.

Supported by grants from the Swedish Medical Research Council no. 19X 580.

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ing similar to type I fibres (II C') to fibres staining similar to type II A fibres (II C'). To the finding that the percentage of type II C fibres was increased after "anaerobic" training but not after "aerobic" training one must assume that the reversible transformation between type I and type II C is faster than the reversible transformation between type II C and type II A fibres. Our findings concerning the possible transformation of type I to type II fibres were supported by recent observations on a well-trained cross-country skier in whom the percentage of type I fibres decreased from 81% to 57% during weeks of immobilization due to an injury (Jansson, Eriksson and Häggmark, personal communication 1978). Thus it might be assumed that not only specific training stimulus but inactivity might induce a conversion of type I to type II muscle fibres.

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TABLE I. Percentage of the different fibre types after aerobic and anaerobic training.

Subject	"Aerobic" training					Anaerobic training				
	I	II A	II B	II C	II B/II A	I	II A	II B	II C	II B/II A
1 GW	60	22	18	0	0.8	34	22	29	15	1.3
2 TB	33	15	1	1	0.1	77	7	4	12	0.6
3 CI	68	30	2	0	0.1	48	16	29	7	1.8
4 MH	66	14	20	1	1.4	48	18	11	14	0.4
Mean	69	20	10	1	0.6	52	18	18	12	1.0
SD	10	7	10	1	0.6	18	9	12	4	0.6

and $\#$ denotes significant difference between the aerobic and anaerobic training at the level of $p < 0.05$ and $\# < 0.01$ respectively

for myofibrillar ATPase (Brooke and Kaiser 1970) to identify the fibre types. 300 (180-480) fibres were classified from each biopsy

After "anaerobic" training, all subjects had a lower percentage type I and a higher percentage type II C fibres than after "aerobic" training. Three subjects also had a higher percentage type II A + II B fibres and an increased II B/II A ratio after "anaerobic" training (Table I). Thus, it seems reasonable to assume that a conversion of type I to type II C fibres had occurred in subjects 1 and 2 due to "anaerobic" training and that a conversion in the opposite direction had occurred in subject 3 and 4 due to "aerobic" training. The type II fibres might be fibres in transformation from type I to type II A or in the reversed direction as hypothesized in Fig. 1. Support for this hypothesis might be found in the fact that there is a continuity in the stain intensity within the type II C fibre population (Fig. 2) from fibre

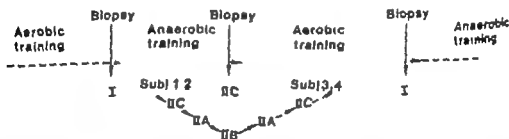


Fig. 1. A hypothetical model for fibre type transformation induced by training



Fig. 2. Serial sections from vastus lateralis (subj. 1, anaerobic training) stained for myofibrillar ATPase after preincubation at pH 10.3 (A), pH 4.6 (B) and pH 4.3 (C). Type II C fibres with stain intensity similar to type I fibres (II C⁺) and to type II A fibres (II C⁻) are present.

1. Age, weight, maximal oxygen uptake, muscle fiber area and fiber type distribution in untrained, oil education students and endurance-trained subjects. V bars are the means for each group.

Age	Number of Subjects	Age (y)	Weight (kg)	Maximal oxygen uptake (ml kg ⁻¹ min ⁻¹)	Muscle fiber area (mm ²)	Mean fiber type distribution			
						Type I	Type II	A	B
Untrained	1649	23 (21-31)	76.4 (63.5-95.0)	50.8 (44.4-57.7)	4266 (2431-6304)	42.4 (22.3-60.7)	57.2 (31.1-50.8)	18.7 (0.0-36.1)	5.7 (0.0-14.6)
Oil education students	339	25 (19-33)	74.4 (64.5-82.0)	62.6 (57.5-66.0)	4305 (3313-5224)	50.9 (42.1-62.7)	49.1 (21.5-52.8)	11.9 (0.0-16.9)	5.6 (0.0-10.1)
Endurance- trained	1739	24 (18-34)	70.1 (58.7-75.5)	74.2 (58.7-79.9)	4409 (3445-6834)	67.6 (41.9-84.1)	32.1 (10.9-42.5)	6.1 (0.0-17.1)	6.2 (0.0-9.2)

Number of subjects (n); Range.

Another factor which may contribute to the dispersion in individual CA values seen in Fig. 1a and b is the relatively large individual differences in fiber type composition (Table 1), since there is a clear difference in CA for the various fiber types (Andersen and Heonson 1977; Ingjer 1978). These differences in CA caused by the large individual variations in fiber area and fiber type composition become much smaller when the mean values for each group is used (Fig. 1). This is caused by the relatively small differences in the percentage

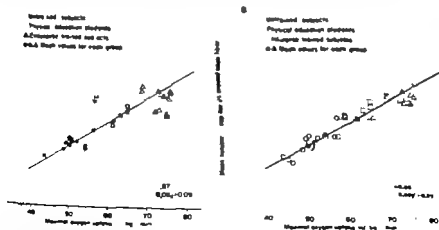


Fig. 1a. Diagram showing relation between mean number of capillaries around each fiber and maximal oxygen uptake (ml kg⁻¹ min⁻¹). Each point represents values from one subject.

Fig. 1b. The same relation as in Fig. 1a, but the individual CA values are here corrected for differences in fiber area between the subjects.

Maximal aerobic power related to the capillary supply of the quadriceps femoris muscle in man

By

FRANK INGJER

The capillary supply of the quadriceps femoris muscle in man has been the subject of several investigations recently (e.g. Andersen and Henriksson 1977 Brodal *et al* 1977 Ingjer and Brodal 1978). Separate untrained and endurance-trained groups (Andersen 1975, Brodal *et al* 1977 Saltin *et al* 1977 Ingjer and Brodal 1978) or one group before and after training (Andersen and Henriksson 1977) have been studied. These studies show that increased maximal aerobic power is accompanied by increased capillary supply. However, since the groups and the range in maximal oxygen uptake are small in these studies, they do not elucidate the type of relationship between these parameters. This requires a large number of subjects with a continuous variation in maximal oxygen uptake ranging from low to high values. Accordingly the present investigation was undertaken to study the relationship between capillary supply and maximal aerobic power in such a group of male subjects.

In two previous studies (Brodal *et al* 1977 Ingjer 1978) the mean values for the capillary supply of the vastus lateralis of the quadriceps femoris muscle in untrained and endurance-trained groups have been given. The individual values from untrained and endurance-trained subjects together with capillarization values from 9 physical education students with maximal aerobic capacity in between the two other groups is presented in this study (Table I). The methods used for taking the biopsies, and for determination of the maximal oxygen uptake, the capillarization values, the fiber areas and the fiber type composition, have been published earlier (Brodal *et al* 1977 Ingjer 1977) and will not be described here.

Fig. 1 shows that there is an approximately linear relationship between the average number of capillaries around each fiber (CA) and the maximal oxygen uptake. There are no statistical differences in fiber areas between the three groups (Table I) and when individual values are examined, there is no relation between fiber area and maximal oxygen uptake. However, individual variation in fiber area (Table I see range) may in extreme instances cause 20-25% difference in CA between subjects with identical maximal oxygen uptake, as calculated from the relationship between fiber area and CA given by Ingjer (1978). This fact explains part of the dispersion of individual values seen in Fig. 1 a, as shown in Fig. 1 b where correction has been made for individual differences in muscle fiber area. A chosen muscle fiber with an area of 4 400 μm^2 and an increase on 0.56 capillaries around each fiber for each 1 000 μm^2 increase in fiber area (Ingjer 1978) has been used.

1. Age, weight, maximal oxygen uptake, muscle fiber area and fiber type distribution in untrained, all education students and endurance-trained subjects. Values are the mean for each group.

	Number of fibers	Age (y)	Weight (kg)	Maximal oxygen uptake (ml kg ⁻¹ min ⁻¹)	Muscle fiber area (mm ²)	Mean fiber type distribution			
						Type I	Type II	A	B
									Other
untrained	1349	21	76.4 (63.3-93.0)	30.8 (44.4-57.7)	4266 (2631-6304)	62.4 (22.3-60.7)	33.2 (21.1-50.8)	18.7 (0.0-36.1)	5.7 (0.0-14.6)
all education students	829	23 (19-33)	74.4 (64.5-82.0)	62.6 (37.5-66.0)	4105 (3313-5224)	30.9 (4.1-62.7)	31.6 (21.5-52.0)	11.9 (0.0-16.9)	3.6 (0.0-10.1)
endurance-trained	1739	24 (18-34)	70.1 (58.7-73.5)	74.2 (58.7-79.9)	4409 (1683-6854)	67.6 (43.9-84.1)	20.1 (10.9-42.5)	6.1 (0.0-17.1)	6.2 (0.0-9.2)

Number of subjects: () Range.

Another factor which may contribute to the dispersion in individual CA values seen in Fig. 1 a and b is the relatively large individual differences in fiber type composition (Table I, etc), since there is a clear difference in CA for the various fiber types (Andersen and Henneson 1977 Ingjer 1978). These differences in CA caused by the large individual variations in fiber area and fiber type composition become much smaller when the mean values for each group is used (Fig. 1). This is caused by the relatively small differences in the percentage

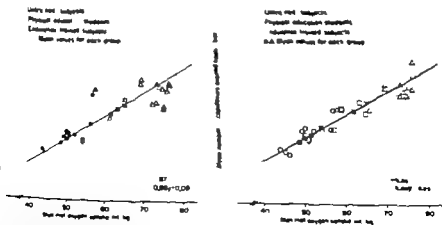


Fig. 1 a. Diagram showing relation between mean number of capillaries around each fiber and maximal oxygen uptake (ml kg⁻¹ min⁻¹). Each point represents values from one subject.

Fig. 1 b. The same relation as in Fig. 1 a, but the individual CA values are here corrected for differences in fiber area between the subjects.

number of type I + II A fibers between the various groups, and the fact that type I and II have both high capillary supply while only type II B has low capillary supply (Andersen and Henriksson 1977 Ingjer 1978), and because a previous study (Ingjer 1979) shown that a $1\,000\ \mu\text{m}^2$ increase in fiber area increases CA by approximately 0.56. In this difference in mean fiber composition can only account for a 3–5% increase in the CA for the untrained to the endurance trained group in this study and the difference in mean fiber area only for 1–3% increase, while the real difference is about 46%. This increase in capillary supply with increasing maximal oxygen uptake corresponds reasonably well with results in oxidative enzyme activities (e.g. Costill *et al* 1976, Bylund *et al* 1977), mitochondrial content (e.g. Gollnick *et al* 1971 Hoppeler *et al* 1973 Bylund *et al* 1977), and capillary area from other studies (e.g. Andersen and Henriksson, 1977).

In conclusion, the present investigation suggests that the capillary supply of the vastus lateralis of the quadriceps femoris muscle in man increases linearly with increasing maximal oxygen uptake, and that the greater part of this increase can be accounted for by the effect of endurance training, and only to a very limited extent by differences in fiber type composition and fiber area between the groups. This points to the capillary supply as one important factor for the aerobic work power.

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Anticholeretic effect of somatostatin in anesthetized dogs

By

ÅKE HOLM, LARS THULIN, HANS SAMDALÅRD, SUAD EFENDIC and GUNNAR TYDÉN

tetradecapeptide somatostatin, originally found to be an inhibitor of growth-hormone-releasing factor has been demonstrated to be active in the gastrointestinal tract. It thus blunts gastrin-induced gastric acid and secretin-induced pancreatic juice (Review Efendic 1978). An inhibiting effect on hepatic bile output would therefore be expected. The present paper is a preliminary report on this subject, which appears not to have been studied previously.

Material and methods

The study was performed in 5 mongrel dogs, weight 30-40 kg. Anesthesia was induced and sustained with pentobarbital sodium and nitrous oxide in oxygen. The animals were endotracheally intubated and ventilated by an Engstrom respirator. Arterial blood gases and acid-base status were controlled regularly and corrected when necessary. Via a small subcutaneous incision, the cystic duct was ligated and a common duct fistula created close to the duodenum. The bile output was measured by weighing each three-minute fraction of bile with an accuracy of 0.01 g. Intra-arterial blood pressure was measured by a catheter located in the abdominal aorta, inserted via the femoral artery and connected to a mechano-electrical recorder. Statistical analysis was performed by the use of Student's *t*-test for independent groups.

Somatostatin (Kabi) was administered as constant rate i.v. infusions in the following manner: I. Three infusions, dosage $0.2 \mu\text{g kg}^{-1} \text{ min}^{-1}$ duration 9 min. II. Four infusions, dosage $1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ duration 9 min. III. Nine infusions, dosage $1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$ duration 1 min.

Results

15-minute infusions

Bile output decreased following somatostatin. Maximum decrease varied interindividually between 40 and 95 per cent after the $0.2 \mu\text{g}$ dose, and between 45 and 85 per cent after the $1.0 \mu\text{g}$ dose. Significant reduction of bile output occurred 6 min after onset of infusion, and maximum effect was obtained 9-12 min after onset of infusion. The bile output remained at a significantly depressed level during 12 min after termination of infusion. There was no change in mean arterial blood pressure.

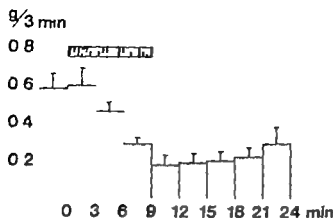


Fig. 1

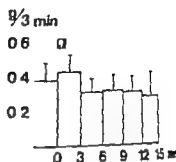


Fig. 2

Fig. 1 Bile output following i.v. infusion of somatostatin. 7 infusions, dosage $0.2-1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$ duration 9 min. Vertical lines indicate S.E.M. $p < 0.05$ $p < 0.01$

Fig. 2. Bile output following i.v. infusion of somatostatin. 9 infusions, dosage $1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$ duration 1 min. Vertical lines indicate S.E.M.

There was no statistically significant difference between low or high dosage in respect to onset or duration of the response. Bile output values for the two groups together are presented in Fig. 1.

One-minute infusions

The bile output did not change following one minute infusions of somatostatin, $1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$. A slight decrease of bile output, observed in most experiments, was not statistically significant. Mean arterial pressure was unchanged. Bile output values are presented in Fig. 2.

Discussion

The present work demonstrates that somatostatin is an anticholinergic agent. The $0.2 \mu\text{g kg}^{-1} \text{ min}^{-1}$ dosage was chosen since it is known to inhibit gastric acid and pancreatic juice secretion. Obviously this dosage has also a maximum inhibiting action on hepatic bile output, equal to that obtained following the $1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$ dosage of equal duration. The anticholinergic effect seems to be more related to the duration of infusion than to the dosage administered. The 9-min infusions at the lower dosage, $0.2 \mu\text{g kg}^{-1} \text{ min}^{-1}$ were more efficient than 1-min infusions at the higher dosage, $1.0 \mu\text{g kg}^{-1} \text{ min}^{-1}$. The minor effect of brief infusions appears to be due to a rapid inactivation of the substance. A calculation based on the duration of the effects, suggests that somatostatin has a half life time of between 2 and 3 min under the present circumstances. This value is in accordance with earlier findings in man (Ekelund unpublished data).

The anticholinergic effect of somatostatin shows a typical slow pattern. This is in contrast to all other effects of the substance, which are immediate. It is also different from the effect of Substance P which is the only physiological substance previously known to inhibit bile output. Substance P is believed to exert a direct inhibitory action on sodium depe-

cular bile output (Holm *et al* 1978). The delayed effect of somatostatin suggests that an indirect action, inhibiting gut hormone-induced bile secretion. This mechanism is further studied.

Summary

Somatostatin, 0.2–1.0 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ was administered i.v. in 5 anesthetized dogs. Following infusions, hepatic bile output was found to decrease by approximately 50% after a γ of 6 min. The mode of action suggests that somatostatin inhibits hormone-induced output.

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The effect of food ingestion on circulating neurotensin-like immunoreactivity (NTLI) in the human

By

M. L. MASHFORD, GÖRAN NILSSON ÅKE RÖKAELIS and SUNE ROSELL

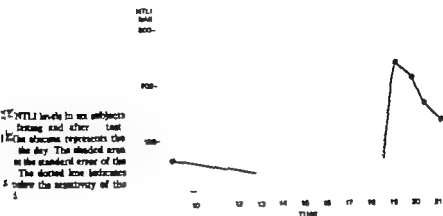
Neurotensin is a tridecapeptide isolated from the bovine hypothalamus and the small bowel (Carraway and Leeman 1973 1976). Immunohistochemical studies have shown that the gut contains glandular cells reacting with antibodies to neurotensin (Orri *et al.* 1977 Sundler *et al.* 1977 Helmsteadler *et al.* 1977). In anesthetized dogs higher concentrations of NTLI have been found in blood draining from the small intestine than in the arterial blood (Rosell *et al.* 1978). Thus the experimental data obtained so far suggest that neurotensin may be a hormone which is stored in and released from the small intestine. One requirement for a circulating material to be regarded as a hormone is that its concentration in blood varies in a reproducible way in relation to some physiologically important event. This study reports the effects of fasting and food intake and also of exercise on peripheral venous concentrations of NTLI in humans. During fasting water was taken *ad libitum*. The subjects were 6 healthy males aged from 23 to 47 years.

Blood was collected into chilled heparinized tubes and spun at 4°C. The plasma was stored at -20°C until subjected to radioimmunoassay for NTLI. The antibody (B-7) used in the assay was raised in rabbits. Details about the method will be reported elsewhere.

The antibody reacts with neurotensin (NT), NT (1-12), (Gln⁴) NT and (Gln⁴) NT (1-12) but not with NT (4-13) or smaller C-terminal fragments.

It shows effectively no cross-reactivity with VIP, secretin, cholecystokinin-33, cholecystokinin-39, pancreatic glucagon, substance P, insulin, somatostatin, GIP, gastrin-17, gastrin or with trypsinized gastrin-34 which presumably contains the N-terminal heptadecapeptide. The assay can measure levels down to 10 pM.

The mean concentration of NTLI in 6 subjects after an overnight fast was 65 ± 12 pM ($M \pm S.E.$) (Fig. 1). Continued fasting was associated with further fall in blood concentration which for 3 of the subjects resulted in concentrations below the sensitivity of the assay. At 1830 h a test meal consisting of an aperitif of 40 ml whisky, white bread roll and 40 g butter, steak, fried potatoes and béarnaise sauce, 100 ml wine and coffee was commenced. All post prandial concentrations of NTLI were significantly elevated. The mean concentration at 45 min was 233 ± 86 pM. The NTLI concentration fell thereafter but remained elevated in all subjects for the 2 h and 45 min during which sampling continued. In another experiment, two of the subjects fasted overnight. Blood samples were taken during the morning and at 1900 h 40 ml whisky were consumed and blood concentration continued at 1930 h.



intervals for 1 h. No significant change in blood concentration of NTLI was seen but a light snack consisting of a sandwich and 100 ml yoghurt taken at 1500 h, a pronounced increase of NTLI occurred. Two subjects fasted overnight and drank 100 ml black coffee at 1200 h. There was no response of NTLI to coffee. Three of the subjects in varying degrees of physical training ran for 40 min at close to each individual's maximal capacity judged by the pulse rate. There was no consistent change in the blood concentration of NTLI.

The data indicate that the ingestion of food appears to exert an overriding influence on the blood concentrations of NTLI. The meals consumed contained fat, proteins, carbohydrates and the influence of distension of the esophagus and the stomach may also be of importance. Alcohol and coffee which were also consumed, did not seem to greatly influence the level when taken alone.

The NTLI cannot be termed neurotensin since it has not been isolated and chemically characterized. Moreover experimental data indicate that some of the actions of neurotensin may be caused by substance(s) formed from neurotensin (Rosell *et al.* 1978). Since the antibody is directed towards the N-terminus of the neurotensin molecule shorter N-terminal peptides of neurotensin may have been detected. The consistent pattern of change in the blood concentration of NTLI associated with food intake suggests that the circulating peptide or peptides are subserving some hormonal function in relation to eating but the nature of such regulatory function is not known. The most sensitive target organs for neurotensin so far are the upper gastrointestinal tract and the adipose tissue. Thus, i.v. infusion of neurotensin in anesthetized dogs ($5-30 \text{ pmol kg}^{-1} \text{ min}^{-1}$) inhibits motor activity in the antral part of the stomach (Andersson *et al.* 1977), inhibits gastric acid secretion (Andersson *et al.* 1976), increases the blood flow in the gastrointestinal tract and has a vasoconstrictor action in subcutaneous adipose tissue but does not appreciably change blood pressure (Rosell *et al.* 1976). At higher doses the blood glucose concentration is elevated (Carraway, Jensen and Leeman 1973; Rosell *et al.* 1976).

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Addendum

During preparation of this manuscript Besterman *et al.* (The Lancet, pp. 785-788, 1978) reported that a test-meal increases the concentration of neurotensin in blood.

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Failure of buccal stimulation with NaCl to evoke an homeostatic response related to body sodium

By

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Pharyngeal receptors are believed to be involved in the control of water and salt intake of water excretion (Epstein *et al.* 1973). Specifically Nicolaidis (1969) working with anesthetized rats, has shown that buccal stimulation with tap water increased urine flow within 1 min whereas buccal stimulation with 5% NaCl solution decreased urine flow within 1 min. We deemed it pertinent to determine whether or not oro-pharyngeal receptors were also involved in the control of Na excretion as are some post-pharyngeal receptors (Daly *et al.* 1967 Carey *et al.* 1976).

Adult Sprague-Dawley rats were anesthetized with Inactin (Promonta) 120 mg/kg i.p. (animals) or with amobarbital 150 mg/kg i.p. (2 animals) with additional small doses. Incisions, one femoral vein and one femoral artery were cannulated for urine sampling, infusion of saline (0.85%) or 5% mannitol on saline, and the recording of blood pressure. On the rat on its abdomen the tongue was fixed in a plastic cup. From a tube the NaCl solution (20 drops/min) ran over the tongue into the buccal cavity and out of the mouth. In 3 expts. the left kidney was denervated by pedicle stripping and application of mol crystals and in one of these animals vasopressin was infused for the last half of the expt. Experimental observations were made in hydropenia (no femoral vein infusion) and following volume expansion (3 to 5 ml of 0.85% NaCl i.v. followed by the same or 5% mannitol in saline at 4 to 6 ml/h). Animals were kept at about 37°C body temperature by a temperature-controlled heating pad.

The results on Inactin anesthetized animals were as follows. In one hydropenic animal with both kidneys intact, 1 M NaCl on the tongue (4 trials) caused complete cessation of urine flow during or immediately following the application. In an additional hydropenic animal with one kidney denervated, dripping 0.5 M NaCl onto the tongue for 15 min (3 trials) appeared to decrease Na excretion during and afterward in both innervated and denervated kidneys (Fig. 1 lower left). After volume expansion in two other rats 0.5 M (2 trials) and 1 M NaCl (2 trials) had little effect on the innervated side and may have caused slight reduction in Na excretion on the denervated side (Fig. 1 upper left). During vasopressin (ADH) infusion, 1 M NaCl on the tongue (1 trial) appeared to be without effect. In the amobarbital anesthetized animals the results were quite different. Urine flow and Na excretion were much greater and 0.5 M NaCl and 1 M glucose were without effect on

Addendum

During preparation of this manuscript Besterman *et al.* (The Lancet, pp. 785-788, 1978) reported that a test-meal increases the concentration of neurotensin in blood.

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Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men

By

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Abstract

ÖRLANDER, J., K. H. KIESLING, L. LARSSON, J. V. KARLSSON and A. ANIANSSON. Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men. Acta physiol. scand. 1978. 104 249-261.

In order to find out if there are age-related changes in human skeletal muscle metabolism or ultrastructure, my material from 56 sedentary men, aged 22-65 years, was studied by means of enzyme activity determination, histochemistry and quantitative electron microscopy. For comparison, younger (16-48 years) and older (66-76 years) groups were included. These subjects were relatively more active. There was a decrease in percentage of slow twitch fibres with age. Mitochondrial volume fraction decreased with age, mainly due to diminished mean mitochondrial volume. In spite of this, no overall decrease in the activity of five enzymes representative of the major pathways in energy metabolism, as observed. Thus, increased amounts of enzymes per unit mitochondrial volume are implicated. Lipofuscin, as more frequently found in the older groups. Correlations were present between fibre type distribution and creatine kinase, as well as between different enzymes. It was concluded, that the decrease in muscular oxygen uptake and muscular strength in aging humans probably may not be explained in terms of deteriorating skeletal muscle energy metabolism.

Key words: aging, muscle metabolism, muscle ultrastructure.

It is a well-known fact that the physical performance capacity will decline during middle age and senescence. The maximal oxygen uptake capacity, the common measure of fitness, will decrease (Åstrand 1960), as well as the muscular strength (Ulfand 1933, Fisher and Berth 1947, Astrumson and Hoebell-Nielsen 1961). It is still unsettled to what extent these effects are due to biological aging or to decreasing physical activity during old age. Training may counteract the physical decline (e.g. Grimby and Saltin 1966), although not completely, indicating that both decreasing physical activity and biological aging are involved.

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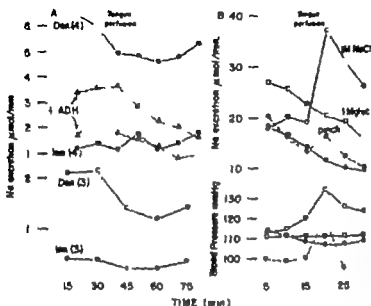


Fig. 1 Effect of 0.3 and 1 M NaCl, 1 M glucose, and tail pinch on Na excretion in conscious (left) and anesthetized (right) rabbits.

blood pressure or Na excretion (Fig. 1 right side). However, 1 M NaCl on the initiated chewing and tongue withdrawal movements, a rise in blood pressure, and a rise in Na excretion. Pinching the tail (5 min) evoked an approximately equal rise in blood pressure but a lesser increase in Na excretion. A second animal gave similar results.

The results are believed to show that whereas 1 M NaCl may elicit either a reduction in urine flow and Na excretion or an increase, depending on experimental conditions, neither of these responses may be considered as specific homeostatic responses used to compensate changes in body sodium. The cessation of urine flow and occasional reduction in Na excretion were presumably the result of release of endogenous vasopressin whereas the increase in Na excretion seen in the amobarbital anesthetized animal was probably related to the arousal engendered by the hypertonic salt. Tail pinch seemed to have a similar effect.

In conclusion, these studies provide no evidence for oro-pharyngeal receptors for Na. They are involved in controlling Na excretion by the kidney.

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TABLE 1. Anthropometrical characteristics of the subject groups. Values are means \pm S.E., and ranges are given within brackets. * and ** denote $p < 0.05$ and $p < 0.01$, respectively when compared with the reference group (20-29).

group	Number of subjects	Age (years)	Height (cm)	Weight (kg)
20-29	6	27 \pm 0 (16-38)	179 \pm 2 (174-184)	70.6 \pm 3.4 (61.2-79.0)
30-39	11	26 \pm 1 (22-29)	182 \pm 2 (170-190)	76.2 \pm 3.2 (58.1-93.0)
40-49	16	34 \pm 1 (30-39)	181 \pm 1 (172-194)	82.8 \pm 3.0 (64.7-108.7)
50-59	11	43 \pm 1 (41-48)	182 \pm 2 (174-190)	80.5 \pm 4.2 (62.4-111.5)
60-69	9	54 \pm 1 (51-57)	180 \pm 2 (171-187)	81.6 \pm 2.5 (74.5-96.8)
70-79	9	61 \pm 1 (60-65)	175 \pm 1 (169-180)	74.4 \pm 3.6 (61.8-96.7)
80-89	7	70 \pm 1 (66-76)	172 \pm 2* (164-180)	77.0 \pm 2.3 (67.0-87.0)

3-hydroxyacyl-CoA dehydrogenase, HAD (E.C.1.1.1.35); the citric acid cycle by citrate synthase, CS (E.C.4.1.3.7), and the respiratory chain by cytochrome oxidase, cyto (E.C.1.9.3.1).

Isosugars were prepared and assays performed as described previously (Örskov *et al.* 1977). PFK activity was determined by the method of Shoenk and Borer (1964), LDH and HAD according to Bass *et al.* (1969), CS as described by Sore (1969) and cyto according to Wherret *et al.* (1969). Protein was assayed by the method of Lowry *et al.* (1951).

Electron microscopy. Small pieces of muscle tissue are processed for electron microscopy and micrographs were taken for morphological estimates according to Weibel (1969). Details of the procedure have been described before (Örskov *et al.* 1977). Since the size distribution and average stage of skeletal muscle mitochondria are sex specific, the composition of mitochondrial number and mean volume in fibres is sex-specific (Weibel 1969; Örskov *et al.* 1977). This means that these values are only rough estimates of the true ones. Because the electron microscopical technique is extremely time-consuming, this part of investigation is limited to 43 subjects.

Statistics. Linear regressions and correlation coefficients were calculated by standard procedures. The significance of correlation coefficients was determined with test T. Test differences between group means, Student's *t*-test was applied, $p < 0.05$ being considered significant.

Results

In Fig. 1-4, group mean values are given at the respective group mean ages, along with regression lines, based on individual values. Regression lines were calculated from values of the 20-65 year groups only. Where no significant correlation was obtained, the best fitting regression line was still indicated (broken line).

Fibre type distribution. The mean percentages of ST fibres in the various groups are given in Fig. 1. A significant ($r = 0.46$, $p < 0.001$) linear increase in ST fibres with age was observed in the 20-65 year groups, and also when the 66-76 year group was included ($r = 0.44$, $p < 0.001$). No significant differences in fibre type distribution within the FT fibre population were observed (Table II), except for the total lack of C fibres in the oldest group

There is a close connection between the physical performance capacity and the aerobic capacity of the working muscles. As has been shown extensively by numerous investigators during recent years, a change in the level of physical activity will produce corresponding metabolic alterations in the muscles involved (for a review see Holloszy and Booth 1976). With this in mind, the possibility that an age-related deterioration of the muscle metabolism might be a cause for the decreasing working capacity during aging suggests itself.

Rather few reports have appeared on metabolic changes in skeletal muscle during age. Furthermore, most investigators have studied the laboratory rat (Strydom and Gahrn 1970, Rockstein and Brandt 1961, Ermini and Verzár 1969, Ermini *et al.* 1971, Ernster, Szelényi 1972, Schmukler and Barrows 1966, Bass *et al.* 1975). In certain respects the results of these studies are conflicting, and they give no clear picture of the metabolic changes during senescence.

The objective of the present investigation has been to survey some parameters indicating the state of the energy metabolism in human skeletal muscle in relation to age. In order to achieve this, muscle biopsy material from subjects aged 16–76 years has been examined enzymatically, histochemically and electron microscopically.

Material and methods

Subjects. A total of 69 healthy men volunteered to participate in the investigation, each having given informed consent. The major part, 36 men aged 20–65, were recruited from the staff of an insurance company. They were all white-collar workers, engaged in no or light physical activity during their spare time and were thus considered sedentary. 24 of them were identical with the subjects of Örlander *et al.* (1977). Data from muscle strength measurements on this subject population are presented separately (Larsson *et al.* 1978 b). Briefly, they show a decrease in isometric as well as dynamic strength in the oldest groups.

The seven oldest men, 66 to 76 years old, were pensioners with various previous occupations and were comparatively active for their age. The remaining subjects were 6 high school students aged 16–18. All but 1 of these were physically active and participated in various sports. Strength measurements in the younger (unpublished) revealed that these subjects were significantly stronger than sedentary men of the same age.

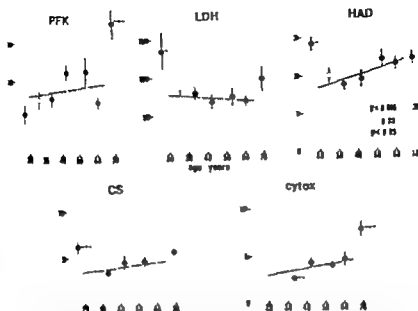
A division into 7 age groups was made with each decade making up one group, except for the youngest and the two oldest groups. The rationale for the deviations from the decade division was that the youngest (16–18) and oldest (66–76) groups were distinct from the others, being non-sedentary and were born before and after the war separately whereas the 60–65 year group resulted from 65 being the age of retirement from the labour company. The 20–29 year group comprising the youngest adult subjects, was chosen as reference group.

Some anthropometrical characteristics of the subject groups are given in Table I.

Muscle biopsies. Muscle tissue was obtained from the vastus lateralis muscle with a needle technique (Bergström 1962). All biopsies were taken at 8–10 a.m. with no preceding exercise. Biopsy material was obtained for histochemistry for determination of enzyme activities and for electron microscopy.

Histochemistry. A muscle piece was trimmed, oriented, mounted in a histoclad, frozen in isopentane cooled by liquid nitrogen and stored at -80°C until analyzed. Serial transverse sections (10 μm) were cut on a cryostat at -20°C , mounted and stained for myofibrillar ATPase (Padykula and Herman 1953, Gauthier and Semmler 1969). Micrographs of the stained sections were taken and fibre classification into slow twitch (ST type I) and fast twitch (FT type II) (Engel 1962) was made in all subjects. In 44 subjects, a further study was made in order to distinguish the a, b and c groups of FT fibres (Brooke and Kaiser 1972, Dubowitz and Brooke 1973). For technical reasons, no histochemical study could be made in the youngest group.

Enzyme assays. To get a picture of the state of the energy metabolism, five enzymes were chosen to represent those for the major pathways. Glycolysis was represented by phosphofructokinase, PFK (E.C.2.7.1.11), lactate fermentation by lactate dehydrogenase, LDH (E.C.1.1.1.27), fatty acid β -oxidation



2. Enzyme activities in various age groups. Activities are given as $\mu\text{mol min}^{-1} \text{kg}^{-1}$ for phosphofructokinase (PFK), lactate dehydrogenase (LDH), 3-hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS), and as $\mu\text{mol O}_2 \text{ min}^{-1} (\text{g et wt})^{-1}$ for cytochrome oxidase (cytox). For further explanations, see legend to Fig. 1. Numbers of subjects (from left to right) 6, 11, 16, 9, 9 and 7. Broken lines indicate non-significant regressions, and dots $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively, have compared with the reference group (20-29).

Cytox was lowered in the 30-39 and elevated in the 66-76 year group ($p < 0.01$ and $p < 0.001$, respectively). No correlation with age was present in the 20-65 year groups. No significant inter-group differences in muscle protein concentration were observed.

When the data from all subjects, regardless of age, were treated together a considerable number of significant correlations between different parameters were observed. The activities of HAD ($r = 0.34$, $p < 0.01$), CS ($r = 0.36$, $p < 0.01$) and cytox ($r = 0.39$, $p < 0.01$) were closely correlated with the percentage of ST fibres (data from 62 subjects). Correlations between enzyme activities, indicating relationships between metabolic pathways, are listed in Table III. The strongest correlations were found between oxidative enzymes. Significant correlations were absent in the pairs PFK-LDH, PFK-HAD and LDH-cytox.

TABLE II. Correlations between enzyme activities in the various age groups. Data from all subjects ($n = 69$) are included in the calculations. PFK, phosphofructokinase; LDH, lactate dehydrogenase; HAD, 3-hydroxyacyl-CoA dehydrogenase; CS, citrate synthase; cytox, cytochrome oxidase; correlation coefficient, r ; not significant, ns.

Enzyme pair									
PFK	PFK	PFK	PFK	LDH	LDH	LDH	HAD	HAD	CS
LDH	HAD	CS	cytox	HAD	CS	cytox	CS	cytox	cytox
0.16	0.04	0.26	0.34	0.27	0.26	0.14	0.73	0.45	0.42
		0.05	0.01	0.05	0.05		0.001	0.001	0.001

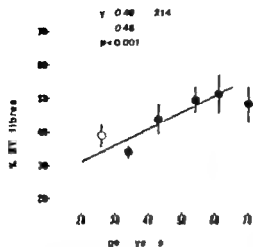


Fig. 1. Percentage slow (ST) fibres in *latissimus* in the 20-76 year groups. Means \pm S.E. each age group are indicated at the respective ages (cf. Table 1). The number of subjects in a group was (from left to right) 11, 14, 11, 8, 9 and 7. The regression line was calculated from the values of the 20-65 year groups. Unfilled \square reference group (20-29).

The histochemical data pertaining to the 20-65 year groups are presented in greater detail separately (Larsson *et al.* 1978 a). Here, they have been included as a background to the metabolic data.

Enzyme activities. Mean activities of the five selected enzymes in the *latissimus* muscles of the various groups are shown in Fig. 2. With the exception of HAD no significant correlations with age were found in the 20-65 year groups.

The PFK activity was significantly higher in the 40-49 and 66-76 year groups than in the reference group ($p < 0.05$ and $p < 0.001$ respectively). For the 20-65 year groups, there was no correlation with age, but when the 16-18 and 66-76 year groups were included, a significant correlation ($r = 0.44$, $p < 0.001$) was obtained.

LDH showed no age differences in the 20-65 year groups. The value of the 16-18 year group was significantly elevated ($p < 0.05$).

For HAD there was a significant ($r = 0.28$, $p < 0.05$) positive linear relationship with age in the 20-65 year groups. This held true also when the 66-76 year group was included ($r = 0.31$, $p < 0.05$). The 16-18 year group's value was significantly elevated ($p < 0.05$) when compared with the reference group.

No significant age differences in CS activity were observed in the 20-65 year groups. The values of the 16-18 and 66-76 year groups were elevated (both $p < 0.001$).

TABLE II. Distribution of fibre types within the fast twitch (type II) fibre population in the 20-76 year groups. Values are means \pm S.E. \square denotes $p < 0.05$ when compared with the reference group (20-29).

Age group	Number of subjects	% of fast twitch fibres		
		Subgroup a	Subgroup b	Subgroup
20-29	6	37 \pm 3	34 \pm 4	9 \pm 3
30-39	11	61 \pm 4	34 \pm 3	5 \pm 2
40-49	7	70 \pm 7	27 \pm 7	3 \pm 1
50-59	7	61 \pm 7	36 \pm 7	3 \pm 1
60-65	8	61 \pm 8	32 \pm 9	7 \pm 2
66-76	7	52 \pm 6	48 \pm 6	0

IV Volume fractions of lipid droplets (means \pm S.E.) and the occurrence of lipofuscin in the various age groups. S.Z. = subsarcolemma zones

group	Number of subjects	Lipid droplets of cell volume	Lipofuscin-containing S.Z. of total number of S.Z.
8	6	0.47 ± 0.16	0 (10) ^a
9	5	0.61 ± 0.13	10 (10)
19	14	0.60 ± 0.10	5 (38)
40	4	1.09 ± 0.42	20 (10)
59	6	0.92 ± 0.18	30 (23)
55	6	0.87 ± 0.32	30 (23)
76	6	0.46 ± 0.16	12 (19)

number of examined S.Z. in each group (num. brackets).

is a significant ($r = -0.36$, $p = 0.05$) negative correlation when the youngest and oldest groups are included. No significant age differences in number of mitochondria per μm^2 sarcomere were observed.

Fig. 4 gives the corresponding data for the subsarcolemma region. There was a linear increase with age in mitochondrial volume fraction ($r = 0.48$, $p < 0.01$) in the 20–65 yr groups. This held true when the values of the 16–18 and 66–76 year groups were included ($r = 0.63$, $p = 0.001$). The values of the three oldest groups were all significantly ($p = 0.01$) less than that of the reference group. A linear decrease ($r = -0.37$, $p < 0.05$) as observed also for the apparent number of mitochondria per unit volume in the 20–65 yr groups. The other groups did, however, not conform to this relationship. No significant age changes in apparent mean mitochondrial volume were present in the 20–65 year groups, but by including the youngest and oldest subjects a negative correlation with age ($r = -0.35$, $p = 0.05$) was obtained.

The volume fraction of lipid droplets in the various age groups is shown in Table IV together with the incidence of observations of lipofuscin (age pigment). There were no significant age differences in lipid droplet content, but some subjects in the 40–65 year groups showed unusually high values. The occurrence of lipofuscin pigment granules, which are localized exclusively in the subsarcolemma region, increased up to 50 years and was then constant. Pigment was most frequently present in fibres with broad Z-lines, presumably ST fibres (Wroblewski and Jansson 1975). Of all observed pigment granules, 71% were found in fibres with broad Z-lines and 15% in fibres with narrow Z-lines, whereas 14% could not be classified. We emphasize, however, that the observations on lipofuscin are a by-product of the present investigation, and the values should be regarded as rough estimates. A systematic study of the subsarcolemma zone is needed for a reliable quantification of lipofuscin occurrence.

When all studied subjects ($n = 43$) were treated together a significant correlation was found between fibrous space and subsarcolemma space mitochondrial volume fractions ($r = 0.44$, $p = 0.01$). No correlations between fibre type distribution and mitochondrial structural data or between mitochondrial structural data and the activities of mitochondrial enzymes were observed.

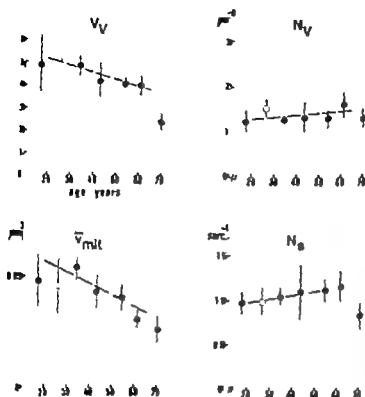


Fig. 3. Mitochondrial volume fraction (V_V), apparent number (N_V) and mean volume (V_{mit}) of mitochondria per sarcomere (N_S) in the sarcolemma space of skeletal muscle fibres at various age groups. Number of subjects (from left to right) 3, 14, 4, 6, 6 and 7. For further explanations, see legends to Fig. 1 and 2.

Muscle ultrastructure All micrographs showed normal gross ultrastructure from visual inspection. The observations on mitochondrial volume fraction, mean volume and number of mitochondria per unit volume or sarcomere in the fibrillar region of most fibres are illustrated in Fig. 3.

The volume fraction of interfibrillar mitochondria tended to decrease with age ($p < 0.1$) in the 20–65 year groups. When the significantly decreased ($p < 0.001$) value of the 66-year group and the value of the youngest group were included, a significant correlation ($r = -0.44$, $p < 0.01$) was obtained. The apparent mean mitochondrial volume showed the same pattern—a tendency ($p < 0.1$) towards an age-related decrease in the 20–65 year group.

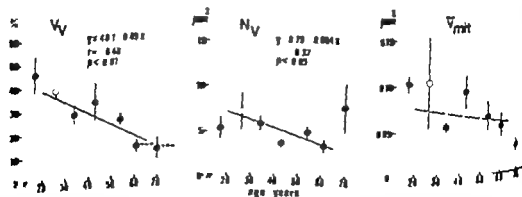


Fig. 4. Volume fraction (V_V), apparent number (N_V) and mean volume (V_{mit}) of mitochondria in the sarcolemma space of skeletal muscle fibres in the various age groups. Number of subjects (from left to right) 3, 14, 4, 6, 6 and 6. For further explanations, see legends to Fig. 1.

younger groups (data not shown), thus supporting this suggestion. The trend towards lower PFK activities with increasing age observed in the present study (Fig. 2) is in line with the findings of Eriksson *et al.* (1973), who found much lower PFK activities in the forearms of 11-13 year old boys as compared to the adult subjects studied by Gofnick (1972). Similarly Ritz and Kirk (1967) observed an age-related increase in PFK activity in human arteries.

The increase in HAD activity with age (Fig. 2), and the similar but weaker trends for aspartate aminotransferase and cytochrome oxidase are easily explained by the parallel increase in ST fibres (Fig. 1), since there are positive correlations between the ST and these enzymes.

It was considered of interest to subject the enzyme activity values to a correlation analysis in order to examine the relationships between metabolic pathways. The observed correlations (Table III) support the validity of the concept of constant proportions between enzymes in different metabolic pathways in muscle (Bass *et al.* 1969). The coordination of mitochondrial oxidative pathways is apparently strong, especially between fatty acid β -oxidation (HAD) and the citric acid cycle (CS) (*cf.* Staudie and Pette 1972). Our results differ somewhat from those obtained by Bylund *et al.* (1976) in human muscle, as they did not observe any correlations in the pairs PFK-CS, PFK-cytochrome oxidase, LDH-HAD or LDH-cytochrome oxidase, but found one between PFK and LDH. It is impossible at present to give an obvious explanation for these discrepancies.

A striking finding in the electron microscopic investigation was that the 16-18 and 66-76 year groups, being different from the others as to relative physical activity level and enzyme activities (Fig. 2), fitted quite well in most trends seen in the 20-65 year groups (Fig. 3-4). This might indicate that there is an endogenous decline in total mitochondrial volume with age, independent—within limits—of the level of physical activity. To maintain the metabolic capacity necessary during sedentary life, or to elevate it in response to physical activity demands during old age, an increase in oxidative capacity per unit mitochondrial volume seems to be required. This might be achieved by an increase in the density of respiratory assemblies on the inner mitochondrial membranes (*cf.* Kjaerling *et al.* 1974, 1975). In contrast, training in younger persons causes the mitochondrial volume fraction to increase in parallel with the oxidative capacity (Örlander *et al.* 1977).

It is further notable that the change towards decreased mitochondrial volume fraction with age proceeded along with an increase in the percentage of mitochondria-rich ST fibres (Fig. 1). This means that on an equal fibre distribution basis, the decline in mitochondrial volume would be even more obvious.

The data in Fig. 3 indicate that the above discussed decrease in mitochondrial volume fraction is caused primarily by a diminished mitochondrial mean volume in the fibrillar space, the number of mitochondria being essentially constant. The pattern is not quite as clear in the subsarcolemmal space (Fig. 4), but if all age groups are included, the same conclusion is reached here. This is partly in accordance with the findings of Kjaerling *et al.* (1973), who in a study of two age groups (mean ages 22 and 57) found an age-related decrease in mean volume with a simultaneous rise in number of mitochondria.

There was a more marked reduction with age in total volume of subsarcolemmal than of interfibrillar mitochondria (Fig. 3-4). It has recently been shown, in cardiac muscle, that

Discussion

The major finding of the present investigation was that no overall decrease in enzymatic activities of skeletal muscle energy metabolism occurs up to 65 years of age in healthy men. Mitochondrial enzyme activities can actually be maintained or even elevated in spite of decreasing mitochondrial volumes.

The question arises, whether systematic differences between the subjects of the different age groups might affect the present results. A hypothetical decline in oxidative enzyme activities (such a decline has been suggested for other organs, see Wilson 1973) could be obscured by a higher physical activity level or a greater percentage of highly oxidative muscle fibres in the older as compared to the younger subjects. Except for the youngest and oldest groups, major differences in physical activity level can be ruled out, since all subjects were more or less sedentary. As to fibre type distribution, there was in the present study a significant increase in the percentage of ST fibres with age (Fig. 1). ST fibres have a greater oxidative potential than FT fibres (e.g. Essén *et al.* 1975), and in the present study poor correlations were observed between the percentage of ST fibres and the activities of the oxidative enzymes (see Results). Calculations based upon the respective regression equations revealed that compensation for the greater proportion of ST fibres in the older groups tended to bring the group mean activities for these enzymes closer to those of the younger group. Thus, there is little reason to assume that differences in fibre composition obscure any decrease in enzyme activities.

Possible causes for the observed change towards more ST fibres with age (Fig. 1) have been discussed elsewhere (Larsson *et al.* 1978a). Some additional comments will be made here.

Fibre type distribution data from studies on untrained subjects by Gollnick *et al.* (1972), KieSSLing *et al.* (1974) and Saltin *et al.* (1976) are in good agreement with the present findings. There is, on the other hand, an obvious dissimilarity with the study by Hedberg and Jansson (1976) on a representative sample of 16-year-old boys (mean 54% ST fibres). The fact that all our groups had lower values might possibly be related to the observation of Hedberg and Jansson that the boys with a low percentage ST fibres were less active and less interested in physical activity than those with a high percentage. It cannot be completely ruled out that the increase in ST fibres seen in the present study might reflect a sample bias towards interest in physical activity in the older groups.

Generally the enzyme activity patterns of the 16-18 and 66-76 year groups deviated from those of the other groups (Fig. 1). The enzymatic profile of the youngest indicated greater anaerobic and fatty acid combustion capacities, no doubt due to their higher physical activity level, whereas the oldest subjects seemed better equipped for aerobic glycolysis when compared with the other groups. The latter finding is hard to explain. These older men were active for their age, participating regularly in activities such as bowling, but when compared with the younger groups there was no great difference. The observed high activities in the aerobic pathway for carbohydrate breakdown may be in some way necessary to maintain the capability of physical activity at this age. Preliminary results from a study in progress of 7 less active men (mean age 71) show PFK, CS and cytochrome activities similar to those of

added that a decrease in aerobic power between the ages of 21 and 38 was due entirely to peripheral factors.

gill's (1963) "error catastrophe" theory of aging states that the accuracy of the cell's synthesizing machinery will deteriorate with age, and the production of more and more error-laden enzyme molecules will eventually cause the death of the cell. The present data, concerning the postmitotic skeletal muscle cell, further add to the body of data indicating no or small changes in enzyme activities during aging in a wide variety of organs and species (Finch 1972, Wilson 1973). Thus, the proposed "error catastrophe" does not seem to markedly affect the amount of active enzyme molecules. There is, however, evidence that there is an increased production of inactive enzyme molecules during aging (Rothstein 1973). This is true for mouse muscle aldolase (Gershon and Gershon 1973). Errors in protein synthesis might thus adversely affect the metabolism of the aging muscle, even though this is not readily apparent from enzyme activity values.

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these two mitochondrial populations differ metabolically (Palmer *et al.* 1977), and our work has shown that they respond differently to e.g. thyroid hormone treatment (Gustafsson *et al.* 1965, Hülsmann 1970, Kubista *et al.* 1971). These observations indicate that the two populations may play somewhat different roles in the muscle cell metabolism. Müller (1976) has suggested that the subsarcolemmal mitochondria supply energy for active transport of metabolites through the sarcolemma, and for protein synthesis. The latter suggestion has also been put forward by Kiessling *et al.* (1973). Furthermore, it is reasonable to assume that the subsarcolemmal mitochondria supply most of the ATP for the active transport of sodium and potassium ions across the sarcolemma. The present results might suggest that some specific function(s) (such as those quoted above), associated with the subsarcolemmal mitochondria, might decline during aging.

Lipofuscin (age pigment, ceroid, lipochrome) granules are thought to be the product of peroxidation of unsaturated lipids present in subcellular organelles (Barber and Bernheim 1967, Chio *et al.* 1969) probably primarily lysosomes (e.g. Frank and Christensen 1970). Accumulation of lipofuscin is a common feature in many aging organs (e.g. Barber and Bernheim 1967, Reichel 1968) including human skeletal muscle (Jermolova *et al.* 1970, Tomonaga 1977), and it has been speculated that peroxidation might be a cause or effect of cellular aging processes involving free radicals (for references, see Barber and Bernheim 1967). Lipofuscin is, however, not unique to aging organisms, since it is found in the newborn human liver (Goldfischer and Bernstein 1969). The present data (Table 1) suggest that there is an increase in lipofuscin occurrence in human muscle up to about 70 years of age, and that the pigment occurs most frequently in ST fibres. The latter observation is consistent with the findings of Shimasaki *et al.* (1977) who found a greater accumulation of pigment in tissues with a prevalence of the 'aerobic' H subunit of LDH, thus supporting the view that *in vitro* oxidation is involved in pigment formation. It is at present not possible to say whether the processes leading to lipofuscin accumulation might adversely affect the functional capacity of the muscle cells.

In conclusion, the results of the present investigation strongly suggest that the decrease in physical performance capacity and muscular strength observed in humans during aging is not explained in terms of a deteriorating skeletal muscle energy metabolism. As judged from the activities of representative enzymes from the major pathways (Fig. 2), the muscle cells of a 65-70-year-old man are still capable of maintaining as great a metabolic flow as those of a young man. It therefore seems improbable, that the decrease in muscular strength should be caused by a diminished supply of ATP (it can, however, not be completely ruled out that there might be an impairment of the coupling between electron transport and phosphorylation during old age). The answer might lie in an impaired function of the contractile apparatus itself: the excitation-contraction coupling, or the neural innervation. Turning to the age-related decrease in maximal aerobic power, the present results speak against a decreasing muscular oxygen utilization capacity as being a primary cause. Thus, an impairment of the oxygen transporting system is implicated. As argued by Aström and Rodahl (1970) this may involve central circulatory (decreasing maximal heart rate, reduced stroke volume) and peripheral (capillary supply?) factors, as well as the efficiency of the regulation of circulation. In a recent longitudinal study Karpman and Ekblom (1978) have

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VAGAL RELEASE OF 5-HT FROM EC

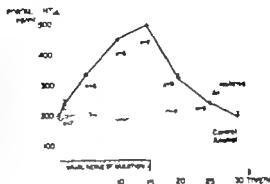


Diagram illustrating portal 5-HT levels in the cat immediately before, during or after electrical stimulation of vagal nerve (mean values \pm S.E.). * indicates that the level after 5 min stimulation is significantly lower compared with the baseline level (Wilcoxon's test). Dotted line indicates the portal 5-HT in control (unoperated but not electrically stimulated).

Recent electrical stimulation of the cervical vagi is known to decrease the tissue 5-HT concentration in the duodenum, as shown by fluorimetric assays (Hohenleitner *et al.* 1971). Instantaneous degranulation of duodenal EC has also been demonstrated by the argentaffin ion (Tansy *et al.* 1971). By use of cytofluorimetry a vagally induced reduction of the cellular levels of 5-HT in EC has been confirmed in the cat (Ahlman 1976). All these findings strongly suggest a vagal release of 5-HT from EC.

In this study was undertaken to simultaneously study the effect of vagal nerve stimulation on 5-HT levels in the portal circulation and on the cellular and subcellular morphology of EC in the cat.

Methods and materials

Surgery, anaesthesia, and blood sampling

Adult cats of both sexes were used. Prior to the experiments the animals were fasted for 4 h but had free access to water. Anaesthesia was induced and maintained with pentobarbitone (30 mg/kg of b.wt.). Blood samples (2.5 ml each) were drawn before, during, and after nerve stimulation according to the time schedule shown in Fig. 1 through heparinized catheters inserted into the portal vein with the tip in the hilar region. Total volume of blood samples from each animal was 20 ml. For volume substitution all animals were given 20 ml of saline solution (0.9%) through the femoral vein before the initiation of electrical stimulation, and an additional 20 ml was given during stimulation. Blood samples were stored on ice during the experiments, centrifuged, and the plasma was kept deep frozen until the assay.opsy specimens of the duodenum were taken from its first portion 1–2 cm apart immediately before ($n=10$) and immediately after onset of nerve stimulation ($n=12$). Full-thickness specimens from 3 animals were prepared for fluorescence microscopy and mucosal specimens were prepared for electron microscopy from all seven stimulated animals.

One animal served as control and therefore had identical operations but no nerve stimulation. Blood samples were drawn following the same time schedule.

Nerve stimulation

Vagi were dissected free at the cervical level and cut. The distal ends were placed in circular electrodes outside of the neck, so isolated from surrounding tissues. A square wave impulse generator (Grass Instruments Co. Model S4) was used for the electrical stimulation. Both vagal nerves were stimulated simultaneously for 15 min, with supramaximal stimulation parameters within the following ranges: 8–20 V, 2 ms, 4–10 Hz. The effectiveness of nerve stimulation was judged by increased bronchial secretion, small vessel constrictions, and urination.

Measurement of 5-HT in plasma

For portal blood samples (2.5 ml) were drawn initially and 1, 5, 10, and 15 min after onset of nerve stimulation, then 5, 10, and 15 min after cessation of stimulation.

The vagal release of 5-hydroxytryptamine from enterochromaffin cells in the cat

By

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Abstract

AHLMAN H., H. N. BHARGAVA, P. E. DONAHUE, B. NEWSON, T. K. DAS GUPTA and L. M. NYHLIN. The vagal release of 5-hydroxytryptamine from enterochromaffin cells in the cat. *Acta physiol. scand.* 1978, 104: 262-270.

Enterochromaffin cells (EC) from the cat duodenum were studied before and after electrical stimulation of the cervical vagi by means of electron microscopy and fluorescence histochemistry according to the Hillarp-Falck technique. From the same animals portal blood plasma was assayed for serotonin (5-HT) at various stages of the nerve stimulation. Within 15 min after the onset of vagal nerve stimulation there was a more than twofold increase of the portal 5-HT levels, which returned to normal after cessation of stimulation. There was also a pronounced reduction in the fluorescence intensity of EC demonstrating lowered intracellular 5-HT level. Ultrastructurally there was evidence for basal exocytosis as well as release of ornithophilic material from the EC directly into the gut lumen after nerve stimulation. Together these observations strongly suggest vagally induced 5-HT release from EC in the cat.

Key words: Vagal nerve stimulation, enterochromaffin cells, 5-HT, cat.

Mammalian gut serotonin (5-HT) is located mainly in the enterochromaffin cells (EC) of the mucosa (Erspamer 1961, Penttilä 1966). In some species 5-HT is present also in the cells (Enerbäck 1973). The presence of 5-HT containing neurons within the myenteric plexus has also been claimed (Gershon *et al.* 1971, 1976). EC are distributed all along the gastrointestinal tract, most frequently in the duodenum (Penttilä 1966, Forsman 1976). They are easily observed by fluorescence microscopy performed according to the Hillarp-Falck technique (1962). Their 5-HT content induces a bright yellow fluorescence at ultraviolet illumination. By the use of cytofluorimetric techniques it now is possible to study intracellular levels of 5-HT in individual EC (Ahlman *et al.* 1976a).



2. Fluorescence micrograph of longitudinal sections from pyloric glands (a) before and after vagal nerve stimulation. (b) Intense yellow fluorescence of EC has been markedly reduced after stimulation. (arrows) In this relative absence of fluorescence, the basal portions of the EC (arrows) after stimulation.

electron-dense core of the granules. Only a few granules had a wide halo between the core and the limiting membrane in the resting state (Fig. 3).

The stimulated EC were partially degranulated. The majority of the secretory granules lost their dense osmophilic core (Fig. 4a), and many granules appeared with diffuse, asymmetrical halos (Fig. 4b). After the nerve stimulation the cytoplasm of the EC frequently was convoluted, developing ocellar-like indentations close to the cell membrane. The secretory granules were frequently associated with these indentations, suggesting an active process of basal exocytosis (Fig. 4c). However it must be emphasized that the response was not all-or-none, and in some sections normal-appearing EC could be seen. Of interest was the unique coalescence of the secretory granules in the apical portion of the cells (Fig. 5). In the sections of stimulated mucosa, a progression of the secretory granules to the most apical part of cells was observed. Also osmophilic material could be demonstrated within the lumen of the gut (Fig. 6).

Discussion

The present study in the cat demonstrates a release of 5-HT into the portal stream during afferent stimulation of the cervical vagi. Such a release could be expected from earlier studies in which a decrease of 5-HT was demonstrated in the duodenum, specifically in the EC, when studied by cytofluorimetry and light microscopy (Tansy *et al.* 1971; Ahlman 1976).

In the fluorescence microscope the major change after vagal nerve stimulation was the marked reduction in fluorescence intensity of the EC, due to a reduction of the intracellular 5-HT, as described earlier by objective cytofluorimetric means in the same experimental preparation after atropinization (Ahlman *et al.* 1976a, b). Occasionally however single cells were seen with a moderately bright fluorescence, suggesting that all EC do not respond

The plasma concentration of 5-HT was determined by the modified fluorimetric method of Ota and Perbach (1973) for brain tissue. Briefly the method consisted of vortexing 250 μ l of plasma with 1 ml n-butanol for 1 min followed by shaking for 5 min on a mechanical shaker at 160 oscillations per min. The mixture was then centrifuged at 3 000 rpm for 5 min at 4 °C. A 2.2 ml aliquot of butanol phase was transferred to a tube containing 5 ml of n-heptane and 1.2 ml of 0.1 M HCl containing 0.1 μ M cyanine II. The mixture was shaken for 5 min at 160 oscillations per minute and then centrifuged at 3 000 rpm for 5 min. 1 ml of aqueous phase was withdrawn and mixed with 0.4 ml of 0.012 M α -phthalaldehyde in 10 M NaOH. The mixture was heated in boiling water for 10 min. After cooling, the fluorescence of the sample was measured at 470 nm after being excited at 360 nm on a Aminco-Bowman spectrofluorometer. The range of 5-HT ranged from 72–81 per cent and the assays were reproducible within 5 per cent.

Fluorescence microscopy

The mucosal biopsy specimens were immediately frozen in liquid isopentane cooled by liquid nitrogen. Thereafter the specimens were freeze-dried for 72 h and then reacted with formaldehyde gas at 30 °C for 1 h according to the Hillarp-Falck procedure (cf. Corrodi and Jonsson 1967). The formaldehyde gas was generated from paraformaldehyde equilibrated at relative air-humidities of 70–90 (Hausegger et al. 1965). After paraffin embedding *in vacuo* the specimens were sectioned at 8–10 μ m and mounted in Epon-Entellane® (1:1) on heated glass slides. Fluorescence microscopy was performed with a Leitz MPB microscope equipped for epillumination, using broad-band excitation with filter combinations described earlier (Malmfors 1965). For photography Tri-X (Kodak) film was used with an exposure time of 60 s.

Electron microscopy

The 4 biopsy specimens from each cat were minced into 8–12 small mucosal specimens, which were immersed in 4% phosphate buffer glutaraldehyde at pH 7.4 for 4 h, while cooled on ice. After rinsing the fixation the specimens were postfixed with 2% phosphate buffer OsO₄ at pH 7.7 for 2 h at room temperature. The specimens were dehydrated in acetones, then embedded in Araldite®. Thick (1 μ m) sections are stained with toluidine blue and used to orientate the sections. Thin sections (600 Å) were cut with a glass knife on a MT-8000 Blum ultramicrotome then counterstained with uranyl acetate and lead citrate and viewed in a RCA EMU 4 electron microscope. Altogether about 250 different cells were studied. However, no morphometric study was not performed.

Results

The efferent electrical nerve stimulation of the cervical vagi resulted in a marked increase of the 5-HT level in the portal vein plasma. Though the stimulation lasted for 15 min, the increase in portal 5-HT was most pronounced during the first 10 min. The increase in portal 5-HT was highly significant ($p < 0.001$) compared with the baseline level already after 5 min of nerve stimulation. With cessation of nerve stimulation a rapid decrease in the 5-HT level was noted after 5 min, reaching baseline levels in 10 min. No changes of portal 5-HT were demonstrated in the nonstimulated, but shamoperated animal (Fig. 1).

When the duodenal specimens were studied by fluorescence microscopy before and after vagal nerve stimulation, distinct morphological changes were seen. The intensely bright yellow EC had, after stimulation, a pale yellow appearance. Furthermore, the 5-HT fluorescence also appeared to have diffused out through the cell membrane. Occasionally a few single cells of moderately high fluorescence intensity were demonstrated in the stimulated specimens. The green fluorescent adrenergic nerve terminals surrounding the intestinal epithelium also appeared to be less intensely fluorescent after nerve stimulation (Fig. 2).

Ultrastructurally the mucosal EC were characterized by the presence of membrane-bound eosinophilic secretory granules in various shapes and varying densities, measuring from 100 nm to 600 nm. A symmetrical clear halo was often seen between the membrane and



Fig. 6. Section cut transversely to the luminal microvilli (mv) of two EC from a stimulated animal. Arrows indicate osmophilic granules in the gland lumen (lum). Note accumulation of characteristic granules in the apical region of both cells (EC₁ and EC₂). er = endoplasmic reticulum, m = mitochondria, d = desmosomes.

in the samples in the same manner. Since the prestimulatory and poststimulatory specimens from the same animal were reacted together in the same vessel during the histochemical procedure, differences in their fluorescence appearance should be true changes and not due to varying reaction conditions.

Ultrastructurally the most significant influence of vagal stimulation was the apparent depagination of these secretory granules. In the resting phase the granules were filled with black or light gray material, but after stimulation the granules were more uniformly light gray or had wide halos, indicating partial release of the osmophilic material. An endocytotic release of secretory material from gut endocrine cells has been earlier described after various types of stimulation (Fujita and Kobayashi 1971; Sano and Tobe 1977).

The fluorimetric assay of 5-HT demonstrated a release of this amine into the portal circulation during nerve stimulation. A direct release of the amine, first into the intercellular space and then to the capillaries, seems probable. However, this study also provides evidence of a vagal release of the osmophilic material directly into the gut lumen as earlier demonstrated for somatostatin (*cf.* Uvnäs-Wallensten *et al.* 1977). If the osmophilic material is identical with 5-HT such luminal release of 5-HT has also been demonstrated in the perfused canine gut at catecholamine-(CA) infusion or splanchnic nerve stimulation by fluorimetric assay of the perfusate (Burks and Long 1966). Since the cervical vagus at the site of the stimulatory electrodes contains CA-nerve fibers running down to the gut (Ahl-

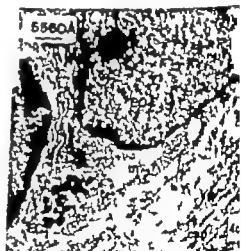


Fig. 3 Ultrastructure of an EC from a nonstimulated animal. Characteristic granules of various shapes and electron densities, often with a thin, symmetrical halo between the dense core and the limiting membrane (arrows). Empty vesicles and granules with wide halos are also seen (double arrows).

Fig. 4 Ultrastructure of EC from the same animal as Fig. 3 after vagal nerve stimulation. (a) EC close to the basal membrane with marked reduction of osmophilia in the granules. Small empty vesicles and wide-halo granules are also seen (double arrows). (b) Detail of another EC higher up in the same gland. Many empty vesicles and wide halos are demonstrated (double-arrows). (c) Detail of an EC close to the basal membrane of another gland. Granules appear relatively unaffected in one of the cells (EC1). However, arrows indicate small clear vesicles and indentations of the outer membrane suggesting local exocytosis in both cells (EC1 and EC2). Note also the apparent increase in endoplasmic reticulum (er) and the swollen mitochondria (m) capillary in interstitial tissue.

Fig. 5 Longitudinal section of an EC from a stimulated animal. Not numerous wide halos among the basally located granules, but also the unique coalescence of osmophilic material (arrows).



Fig. 6. Section cut transversely to the luminal microvilli (lrv) of re-EC from stimulated animal. Arrows indicate osmiophilic granules in the gland lumen (lum). Note accumulation of characteristic granules in the apical region of both cells (EC₁ and EC₂). er = endoplasmic reticulum, m = microvilli, d = desmosomes.

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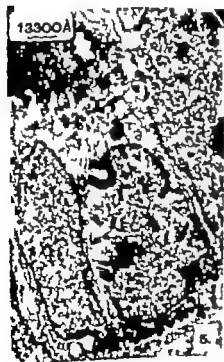


Fig. 3. Ultrastructure of an EC from nonstimulated animal. Ch. recteratic granules of various sizes and electron densities, often with a thin, symmetrical halo between the dense core and the limiting membrane (arrows). Empty vesicles and granules with wide halos are also seen (double arrows).

Fig. 4. Ultrastructure of EC from the same animal as Fig. 3 after vagal nerve stimulation. (a) EC close to the basal membrane with marked reduction of osmophilia in the granules. Single empty vesicles and wide halo granules are also seen (double arrows). (b) Detail of another EC higher up in the same gland. Many empty vesicles and wide halos are demonstrated (double arrows). (c) Detail of two EC close to the basal membrane of another gland. Granules appear relatively unaffected in one of the cells (EC1). However, arrows indicate small clear vesicles and indentations of the outer membrane suggesting basal exocytosis in both cells (EC and EC2). Note also the apparent increase in endoplasmic reticulum (er), and the swollen mitochondria (m). cap - capillary in interstitial tissue.

Fig. 5. Longitudinal section of an EC from a stimulated animal. Note numerous wide halos among the basally located granules, but also the unique coalescence of osmophilic material (arrow). (c) - central part of the cell (arrows).

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man *et al* 1978), the demonstrated vagal release of 5-HT might hypothetically be a C-mediated event. The low fluorescence intensity of the adrenergic nerve terminals after nerve stimulation also suggests that an adrenergic pathway to the gut might have been active (Ahlman *et al* 1976 b). Whether 5-HT is released directly into the bloodstream or whether it is also released into the gut lumen is not clear. If released directly into the lumen only, its prompt rise in portal 5-HT would reflect a rapid absorption of 5-HT from the lumen, perhaps aided by the local vasodilatory effect of the amine (Biber 1973).

The nerve stimulation was effective in all animals, judged by the changes in portal 5-HT levels; however both fluorescence and electron microscopy revealed single cells with granules which seemed to be unaffected. This might be due to local factors, but also suggests the possibility of separate populations of EC. Fluorescence and electron microscopic studies have revealed at least two types of morphologically distinct populations of EC (Vasek *et al* 1971, Gorgas and Böck 1976). Immunofluorescence studies have also demonstrated two populations of EC that store different polypeptides, such as substance P and somatostatin, in addition to 5-HT (Pearse *et al* 1974, 1975). It is therefore possible that such populations may react differently to exogenous stimulation.

A direct innervation of submucosal EC has been reported (Matsuo and Seki 1976). Recently a very close anatomic relationship has been described between nerve terminal dendrites, and the mucosal EC, making a functional neural influence on those cells likely (Lundberg *et al* 1977). The increase of 5-HT in the portal blood after vagal nerve stimulation might therefore reflect a direct neurogenic release. However intraluminal stimulation have been reported to be effective in releasing 5-HT into the portal circulation. Hyperosmolar sugar solutions, food, and duodenal acidification have proved to be effective in this respect (Smith 1938, Drapanas *et al* 1962, White *et al* 1968, Jaffe *et al* 1977). Such stimuli might act directly either on postulated luminal receptors of the EC or on sensory mucosal nerve endings, or cause release of intermediary mediator substances—all possibilities leading to a final release of 5-HT from EC.

5-HT has long been implicated in the physiology of the upper gastrointestinal tract. By using a very sensitive radioimmunoassay Kellum and Jaffe (1976) have demonstrated an increased peripheral blood level of 5-HT after a meal. Biber (1973) earlier had suggested that 5-HT might be the transmitter in a postprandial vasodilation reflex. Other authors have emphasized the importance of 5-HT as a potent inhibitor in the feedback regulation of gastric acid secretion (Resnick *et al* 1962, Wise *et al* 1968, Jaffe *et al* 1977). It may seem somewhat contradictory that a procedure such as vagal nerve stimulation can activate gastric acid secretion and, conversely may lead to its inhibition by the release of 5-HT. However it is possible that the gastric acid and 5-HT responses are mediated via two different types of efferent nerve fibers within the vagus (Ahlman 1976) which are activated simultaneously by the electrical stimulation in a nonphysiologic manner.

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Influence of phenobarbital on changes in the metabolites of the energy reserve of the cerebral cortex following complete ischemia

By

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Abstract

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In the present study which concerns the rate of changes in the cerebral cortex concentrations of phosphocreatine (PCr), ATP, ADP, AMP, lactate and pyruvate during complete ischemia, had the objective of finding out whether or not phenobarbital retards depletion of tissue energy reserves during ischemia. Mice were subjected for periods of 10-20 min to ischemia maintained on 70% N₂O or given 150 mg/kg of phenobarbital. The results showed that the barbiturate anaesthesia delayed utilization of ATP during the first 2 min. However, after 5 min of ischemia PCr and ATP concentrations, as well as the calculated aerobic energy charge, are identical in animals anaesthetized with nitrous oxide and phenobarbital. Thus, phenobarbital induces a very moderate delay in the depletion of cerebral energy reserves that can during complete ischemia. The results obtained after 3-20 min of ischemia allowed calculation of energy (~P) utilization according to Lowry *et al.* (1964). The closed system method gave values for ~P utilization which were not far from those obtained by CMRO₂ measurements. However, with normal rates for metabolic rate (70% N₂O) valid estimates are obtained only with very short ischemic periods (10 s) and, with such short periods, the oxygen content of the tissue may introduce an error.

Although it is usually held that anaesthetics, and notably the barbiturates, retard changes in the metabolites of the energy reserve during complete cerebral ischemia, published results are equivocal. Lowry *et al.* (1964), in their comprehensive study of cerebral metabolic changes during ischemia, noted that phenobarbital slowed down anaerobic utilization of phosphocreatine (PCr) and ATP in 10-day old mice. The authors proposed a method for calculating preischemic energy (~P) flux from the rate of changes in glycogen, glucose, PCr, ATP and ADP (or from PCr, ATP, ADP and lactate) during the first 15 s of ischemia ("closed system method"). Phenobarbital, in a dose of 150 mg/kg, reduced ~P utilization to about 60% of the awake value. Later results from the same laboratory confirmed that phenobarbital delays energy depletion during the first 15-20 s of ischemia (Gatfield *et al.* 1966, Folbergrova *et al.* 1970; see also Brunner *et al.* 1971).

Subsequent studies on dogs have given results that are somewhat in variance with those reported in mice. Michenfelder and Theye (1970) induced ischemia by decapitation in animals under four anaesthetic circumstances, which provided a wide range of values for cerebral oxygen uptake (CMR_{O_2}) and measured rate of changes in tissue concentrations of ATP and lactate. In all groups, there was a linear fall in ATP and a linear rise in lactate concentration during the first four min of ischemia. Irrespective of the type (or depth) of anaesthesia, there was no difference in rate of metabolic changes, but hypothermia (37°C) reduced both the rate of fall in ATP and rate of rise in lactate concentration. On the basis of their results, the authors concluded that anaesthesia and hypothermia reduce metabolic rate by dissimilar mechanisms, and that anaesthetics cannot be expected to protect against the harmful effects of ischemia.

In the present study postischemic alterations in tissue concentrations of PCr, ATP, ADP, AMP, lactate and pyruvate were measured in paralysed rats that were either anaesthetized with 70% N₂O or with 150 mg/kg of phenobarbital, employing ischemic periods varying between 5 sec and 10 min. Since 70% N₂O does not significantly reduce CMR_{O_2} in park rats (Carlsson *et al.* 1976) the results allow evaluation of the "protective" effect of phenobarbital in complete ischemia.

Some of the present results have been reported in a preliminary communication (Nord *et al.* 1975) which was concerned with energy ($\sim P$) utilization, as calculated with the closed system method of Lowry *et al.* (1964). Since this method has been extensively used for estimating metabolic rate in the brain, a somewhat more detailed discussion of the results is given presently.

Material and methods

The experiments were performed on fed male Wistar rats (300–400 g). The animals were divided into two groups. In the first group anaesthesia was induced with 2–3 halothane, allowing tracheotomy. Anaesthesia was continued with artificial respiration using a gas mixture containing 70% N₂O and 30% O₂. In the second group of animals was given 150 mg/kg of phenobarbital *i.p.* After about 10–15 min, when the animals were deeply anaesthetized, they were tracheotomized and artificially ventilated on 70% N₂O and 30% O₂. All animals were immobilized with tubocurarine chloride (1 mg/kg *i.v.*) and were given heparin (100 I.U. *i.v.*).

In all animals a catheter was inserted into one femoral artery to allow continuous recording of arterial blood pressure and anaerobic sampling of blood. Body temperature was kept close to 37°C and arterial PCO₂ and PO₂ were adjusted to 35–40 mmHg and 120–140 mmHg, respectively. A left-sided craniotomy (10–15 mm) exposed the dura over the fronto-parietal cortex. Following operation all animals were kept undisturbed for 20–30 min before the experiments were continued. The exposed cortex was superfused with pre-warmed (37°C) artificial cerebrospinal fluid.

The cerebral cortex was frozen either without or with previous interruption of the cerebral circulation by decapitation, using isopentane pre-cooled to about -150°C in liquid nitrogen, which was allowed to submerge the exposed dura. The period of ischemia, the time between decapitation and freezing of the tissue, was either 5, 10, 20, 60 or 120 s (N₂O anaesthesia) or 10, 20, 60, 120, 300 or 600 s (phenobarbital anaesthesia). Since speed of freezing is not critical, extended periods of ischemia, thus obtained after 5 and 10 min of ischemia in phenobarbital-anaesthetized animals were compared to previous data for animals maintained on 70% N₂O (Ljunggren *et al.* 1974). Control experiments showed that the exposed 1 mm of the cerebral cortex froze within 1–2 s. This part of the tissue was used for the chemical analysis.

The tissue samples were stored at -80°C until extraction. The analyses were performed with specific enzymatic fluorometric techniques (Lowry and Passonneau 1972) as described in detail in previous communications from this laboratory (Folbergrova *et al.* 1972 a, b).

measured organic phosphates (PCr, ATP, ADP, AMP) and substrates (pyruvate and lactate) were used as $\mu\text{mol g}^{-1}$ of wet tissue. The sum of the adenine nucleotides (ΣAd) was calculated as

$$\Sigma\text{Ad} = [\text{ATP}] + [\text{ADP}] + [\text{AMP}] \quad (\mu\text{mol g}^{-1}).$$

Energy state of the tissue was calculated in terms of the adenylate energy charge (E.C.) according to Atkinson (1968):

$$\text{E.C.} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

$\sim\text{P}$ utilization was estimated from changes in PCr, ATP, ADP and lactate according to Gist *et al.* (1966). Since glycogen concentrations did not change significantly in 20 min (unpublished results), calculation should give a good estimate of $\sim\text{P}$ flux. Statistical differences were evaluated with Student's *t*-test.

Results

Tissue concentrations of labile metabolites in animals anesthetized with N_2O or phenobarbital are given in Table I and II, respectively. In general, the results confirm many previous studies in showing that ischemia is accompanied by rapid reductions in the tissue concentrations of PCr and ATP by a progressive rise in lactate concentration, by a transient rise in ADP and by a delayed and massive rise in AMP concentration (see, e.g., Lowry *et al.* 1964, Gattfield *et al.* 1966, Gercken and Preuss 1969, Müller *et al.* 1970, Ljunggren *et al.* 1974). An unexpected result was an increase in the sum of adenine nucleotides after 2 min of ischemia. This occurred when the ADP concentration was maximally increased. Evidently the result is artefactual and is caused by the fact that the method for ADP is not so specific (see Lowry *et al.* 1964).

One finding has not been recorded previously. Thus, whereas Lowry *et al.* (1964) observed rise in pyruvate concentration during the first 100 s of ischemia in 10-day old mice, anesthetized with phenobarbital, the present results fail to demonstrate an increase in pyruvate in adult rats, whether anesthetized with 70% N_2O or 150 mg kg^{-1} of phenobarbital. An initial decrease in pyruvate was recently observed also in 7-day old rats (Hansen and Nordström, in preparation) which may indicate that the rise occurs only in juvenile animals.

The main results of the present study concern differences between the two anesthetic treatments, and calculated $\sim\text{P}$ utilization. These findings will be illustrated separately.

Fig. 1 illustrates the rate of fall in PCr and ATP concentrations (in per cent of control), and the reduction in adenylate energy charge. Phenobarbital anesthesia somewhat delayed the fall in PCr concentration but, after 2 min, the PCr stores were virtually depleted in both groups. With 70% N_2O there was measurable decrease in ATP concentration already after 1 s, and the ATP was less than 10% of control after 2 min. In contrast, animals under phenobarbital-anesthesia showed no fall in ATP concentration during the first 20 s, and about 50% remained after 2 min. However after 5 min ATP was close to zero in both anesthetic groups. The adenylate energy charge was reduced already after 10 s of ischemia in both nitrous oxide and phenobarbital groups. During the first 2 min of ischemia the

Subsequent studies on dogs have given results that are somewhat in variance with those reported in mice. Michenfelder and Theye (1970) induced ischemia by decapitating animals under four anaesthetic circumstances, which provided a wide range of initial cerebral oxygen uptake (CMR_{O_2}) and measured rate of changes in tissue concentrations of ATP and lactate. In all groups, there was a linear fall in ATP and a linear rise in lactate concentration during the first four min of ischemia. Irrespective of the type (or depth) of anaesthesia, there was no difference in rate of metabolic changes, but hypothermia (37°C) reduced both the rate of fall in ATP and rate of rise in lactate concentration. On the basis of their results, the authors concluded that anaesthesia and hypothermia reduce metabolic rate by dissimilar mechanisms, and that anaesthetics cannot be expected to protect against the harmful effects of ischemia.

In the present study postischemic alterations in tissue concentrations of PCr, ATP, ADP, AMP, lactate and pyruvate were measured in paralyzed rats that were either anaesthetized with 70% N_2O or with 150 mg/kg of phenobarbital, employing ischemic periods varying between 5 sec and 10 min. Since 70% N_2O does not significantly reduce CMR_{O_2} in paralytic rats (Carlsson *et al.* 1976) the results allow evaluation of the "protective" effect of phenobarbital in complete ischemia.

Some of the present results have been reported in a preliminary communication (Nordström *et al.* 1975) which was concerned with energy ($\sim P$) utilization, as calculated with the densitometric method of Lowry *et al.* (1964). Since this method has been extensively used in estimating metabolic rate in the brain a somewhat more detailed discussion of the results is given presently.

Material and methods

The experiments were performed on fed male Wistar rats (300–400 g). The animals were divided into two groups. In the first group anaesthesia was induced with 2–3% halothane to allow tracheotomy. Anaesthesia was then continued with artificial respiration using a gas mixture containing 70% N_2O and 30% O_2 . The second group of animals was given 150 mg/kg of phenobarbital *i.p.* After about 10–15 min, when the animals were deeply anaesthetized they were tracheotomized and artificially ventilated on 70% N_2O and 30% O_2 . All animals were immobilized with tubocurarine chloride (1 mg/kg *s.c.*) and were given heparin (100 IU *i.v.*).

In all animals a catheter was inserted into one femoral artery to allow continuous recording of arterial blood pressure and anaerobic sampling of blood. Body temperature was kept close to 37°C and arterial PO_2 and PCO_2 were adjusted to 35–40 mmHg and 120–140 mmHg, respectively. A left-skilled craniotomy (10–15 mm) exposed the dura over the fronto-parietal cortex. Following operation, all animals were undisturbed for 20–30 min before the experiment was continued. The exposed cortex was superfused by pre-warmed (37°C) artificial cerebrospinal fluid.

The cerebral cortex was frozen either without or with previous interruption of the cerebral circulation by decapitation, using isopentane pre-cooled to about -150°C in liquid nitrogen, which was allowed to superfreeze the exposed dura. The period of ischemia, i.e. the time between decapitation and freezing of the tissue, was either 5, 10, 20, 60 or 120 min (N_2O anaesthesia) or 10, 20, 60, 120, 300 or 600 min (phenobarbital anaesthesia). Since speed of freezing is not critical in extended periods of ischemia, values obtained after 5 and 10 min of ischemia in phenobarbital-anaesthetized animals were compared to previous data for animals maintained on 70% N_2O (Ljunggren *et al.* 1974). Control experiments showed that the superficial 1 mm of the cerebral cortex froze in 1–2 s. This part of the tissue was used for the chemical analyses.

The tissue samples were stored at -80°C until extraction. The analyses were performed with specific enzymatic fluorometric techniques (Lowry and Passonneau 1972) as described in detail in previous communications from this laboratory (Folbergrova *et al.* 1972 a, b).

Area: Use lactate/pyruvate ratio during incubation as measure of activity

Area	Use lactate/pyruvate ratio during incubation as measure of substrate utilization											
I. phenanthrene group	PC	ATP	ADP	AM	Ad	LC	La	Py	Lx/Py			
Control	4.94 ±0.12	2.92 ±0.01	0.273 ±0.007	0.042 ±0.002	3.35 ±0.01	0.945 ±0.001	1.15 ±0.24	0.104 ±0.020	12.73 ±0.53			
Incubation (-4)	3.33 ±0.18	2.89 ±0.03	0.363*** ±0.010	0.054 ±0.003	3.31 ±0.06	0.929* ±0.003	1.44 ±0.07	0.080 ±0.009	20.63 ±0.70			
Incubation (-4)	2.27* ±0.07	2.92 ±0.09	0.373 ±0.007	0.054 ±0.002	3.34 ±0.09	0.928 ±0.003	2.79 ±0.07	0.063 ±0.005	44.10* ±2.40			
Incubation (-5)	0.65 ±0.08	2.91 ±0.13	0.704*** ±0.112	0.218 ±0.075	3.44 ±0.03	0.835 ±0.036	7.23*** ±0.53	0.093 ±0.010	79.3** ±10.0			
Incubation (-4)	0.20* ±0.03	1.41 ±0.15	1.420* ±0.070	0.931 ±0.106	3.74 ±0.02	0.563 ±0.058	8.08 ±0.12	0.044 ±0.010	22.1 ±50			
Incubation (-4)	0.66*** ±0.01	0.17*** ±0.02	1.122* ±0.026	1.976 ±0.013	3.27 ±0.06	0.234 ±0.004	10.83*** ±0.13	0.018 ±0.003	63.2** ±90			
Incubation (-4)	0.02* ±0.01	0.07* ±0.00	0.962 ±0.026	1.926 ±0.039	2.97** ±0.06	0.186 ±0.002	12.18 ±0.28	0.003 ±0.000	4.84*** ±366			

TABLE 1 Changes in PCr, ATP, ADP, AMP, lactate and pyruvate as well as in the calculated values for adenine nucleotide pool (cAd), energy charge (E.C.) and the lactate/pyruvate ratio during ischemia in animals anesthetized with \bar{m} -N O

Experimental group	PCr	ATP	ADP	AMP	cAd	E.C.	La	Py	La/Py
Control (n=8)	4.74 ±0.09	2.91 ±0.03	0.294 ±0.013	0.030 ±0.006	3.25 ±0.04	0.940 ±0.003	1.50 ±0.14	0.125 ±0.009	11.89 ±0.41
Ischemia (n=8) 5 sec	3.13 ±0.12	2.74 ±0.03	0.368 ±0.020	0.071 ±0.009	3.15 ±0.05	0.970* ±0.005	2.08 ±0.07	0.103 ±0.008	21.18 ±1.82
Ischemia (n=8) 10 sec	2.35 ±0.13	2.72 ±0.03	0.405 ±0.025	0.076 ±0.012	3.21 ±0.03	0.913 ±0.008	2.76 ±0.16	0.089* ±0.005	31.04 ±1.02
Ischemia (n=4) 20 sec	1.84 ±0.03	2.60* ±0.06	0.397* ±0.014	0.075 ±0.007	3.08 ±0.07	0.911 ±0.004	3.87 ±0.13	0.093 ±0.009	42.5 ±3.3
Ischemia (n=4) 1 min	0.32 ±0.03	1.49* ±0.10	1.176 ±0.069	0.890* ±0.068	3.56 ±0.03	0.585 ±0.031	8.60* ±0.56	0.079 ±0.015	115 ±12
Ischemia (n=4) 2 min	0.06** ±0.01	0.24 ±0.02	1.020* ±0.021	1.999* ±0.036	3.26 ±0.04	0.229 ±0.010	11.08 ±0.49	0.022 ±0.002	517* ±27
Rehearsal	0.05 ±0.02	0.23 ±0.04	0.63 ±0.07	1.93 ±0.04	2.79 ±0.05	0.20 ±0.02	13.99 ±0.63	0.018 ±0.008	4.782 ±1.952
Ischemia	0.09 ±0.003	0.09 ±0.002	0.51 ±0.03	1.88 ±0.04	2.46 ±0.05	0.13 ±0.01	14.03 ±0.63	0.013 ±0.005	3.896 ±2.291

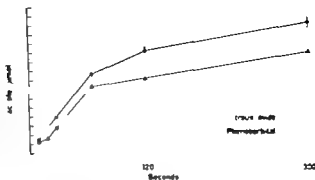
* $p < 0.05$

** $p < 0.01$

$p < 0.001$

Values from LUNDQVIST *et al.* (1974). For these, there was a highly significant difference from control values ($p < 0.001$).

Increase in lactate concentration after 5, 10, 20, 60, 120 sec of ischemia in rats anesthetized with nitrous oxide or urethane. The values are \pm S.E. Filled symbols denote significantly different from controls ($p < 0.05$).



Discussion

Freezing techniques have not been well suited to delineate metabolic changes occurring after short periods of ischemia. This is due to the fact that when animals (or their organs) are immersed in a suitable coolant, there is an inevitable delay before the tissue is frozen (see e.g. Swaab and Boer 1973, Ferrendelli *et al.* 1972, Pontén *et al.* 1973 a, b). Thus the actual ischemic periods are longer than those given, and the error introduced is directly related to the thickness of the insulating layers, and thereby to the size of the animals used. The present technique avoids this artifact since freezing was performed through the exposed dura (the circulation in the cortex being maintained) and since only the superficial 1 mm was used for analyses. It can be calculated that with this technique the tissue analysed is frozen in about 1-2 s. The freezing technique does not alter the levels of any metabolites. The rapidity of freezing is, however, clearly demonstrated in the low activity of phosphorylase α obtained (Folbergrova *et al.* 1978).

Influence of phenobarbital on delaying depletion of energy reserves following ischemia

The present results amply confirm those of Lowry and associates (see Introduction) in showing that phenobarbital retards the rate of fall in energy reserves of the tissue following cerebral ischemia. Thus, whereas animals under 70% N_2O have less than 10% of their ATP content left after 2 min of ischemia, phenobarbital-anesthetized animals still retain about 30% of the ATP pool at that time. However, if ischemia is prolonged to 5 min the energy reserves are depleted of energy whether the animals are anesthetized with 70% N_2O or 150 mg/kg of phenobarbital. It may be asked if the alleged protective effect of the barbiturates against ischemia is at all related to the fact that they delay energy depletion within the first 5 min. In view of the fact that phenobarbital protects against the harmful effects of 30 min of cerebral ischemia although it cannot prevent energy depletion to occur within the first 5 min (Folbergrova *et al.* 1978), it seems necessary to invoke other explanations.

Evaluation of the closed system method for calculating metabolic rate

Since the closed system method was introduced by Lowry *et al.* (1964) it has been used in a number of studies. The results have been quite variable and the method has never been evaluated against another technique for measuring metabolic rate. As discussed in a pre-

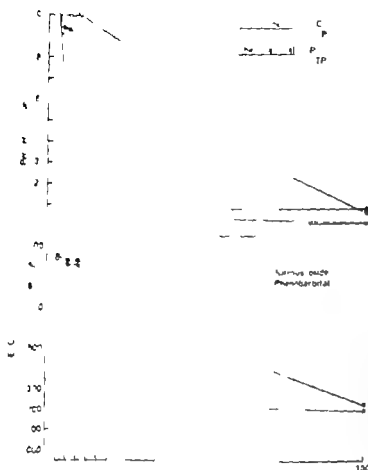


Fig. 1 Changes in energy in the cerebral cortex after 1, 60, 120 and 300 s of ischemia in rats anesthetized with nitrous oxide or phenobarbital. The upper figure shows the fall in PO₄ ATP (mM) and the lower figure gives the change in ADP (mM). Filled symbols denote values significantly different from controls ($p < 0.05$).

energy charge was significantly higher in the phenobarbital-anesthetized animals. However, no significant difference was seen after 5 min of ischemia.

Fig. 2 shows the rate of rise in lactate concentration. With 70% N₂O there was a rise in lactate at a rate of $7 \mu\text{mol g}^{-1} \text{min}^{-1}$ during the first min. Since normal glucose consumption in the cerebral cortex of the rat is $0.8 \mu\text{mol g}^{-1} \text{min}^{-1}$ (Borgström *et al.*), the maximal rate of glycolysis during ischemia represents a 4- to 5-fold increase in control. In the phenobarbital anesthetized animals, the rate of rise was delayed during the first 10 s. However, in the following 50 s period lactate rose as it did in the nitrous oxide animals. Thus, although phenobarbital retarded lactate production initially, it did not retard the peak rate of glycolysis. This finding confirms results reported by Lowry *et al.* (1970). The present results show that somewhat less lactate accumulated under phenobarbital anesthesia than under 70% N₂O (see Table I). In all probability this is due to the fact that in paralyzed animals, phenobarbital anesthesia is usually accompanied by lower blood (tissue) glucose concentrations than is anesthesia with 70% N₂O.

In summary, the present results demonstrate that phenobarbital anesthesia significantly retards utilization of high energy phosphate compounds during the first 2 min of ischemia, while this difference is no longer detectable if the period of ischemia is prolonged to 10 min.

If the mean tissue oxygen tension is supposed to be 35 mmHg (see Mitnick and 1977) the amount of oxygen dissolved in brain tissue should be approximately $0.045 \mu\text{l g}^{-1}$. The total oxygen content of the blood and brain tissue should therefore be $0.045 + 0.11 \mu\text{mol g}^{-1}$. The oxygen utilization rate of the rat cerebral cortex during 1 conditions is $4.5 \mu\text{mol g}^{-1} \text{min}^{-1}$ or $0.075 \mu\text{mol g}^{-1} \text{sec}^{-1}$. Thus, the calculated amount of oxygen in the brain should suffice for 1.2 s of aerobic energy production. This is only of importance when very short periods of ischemia are used for calculation of rate of energy utilization, since the calculation indicates that energy derived from the stores could represent 20–40% of that used during 5 s of ischemia.

It is tentatively concluded that the closed system method of Lowry *et al.* (1964) gives reasonably accurate values for pre-ischemic energy flux when metabolic rate is reduced. At normal metabolic rate, errors introduced by inexact timing of the period of ischemia, by post-ischemic augmentation of metabolic rate, and by the oxygen content of the tissue, tend to render calculated values uncertain. It seems questionable that the method can be applied to situations in which metabolic rate is increased.

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TABLE III Rate of energy utilization in the rat cerebral cortex calculated from the closed system and from CMR_{O_2} measurements.

Anaesthesia	Number of animals	Time after decapitation (seconds)	$\sim P$ utilization ($\mu\text{mol g}^{-1} \text{ min}^{-1}$)	Energy (J) calculated from CMR_{O_2} ($\mu\text{mol g}^{-1} \text{ min}^{-1}$)
N_2O	8	5	30 ± 2	29
	8	10	4 ± 2	
	4	20	17 ± 1	
Phenobarbital	4	10	11 ± 2	15
	3	20	12 ± 1	

In a preliminary communication the present results allow such a validation in that CMR_{O_2} in rat has been measured both in animals under 70% N_2O (Hägerdal *et al.* 1975) and in animals anesthetized with 150 mg/kg of phenobarbital (Nilsson and Siejö 1975).

Calculated rates of $\sim P$ utilization are given in Table III and are compared to those derived from measurements of CMR_{O_2} . In animals anesthetized with 70% N_2O the values calculated after 5 or 10 s of ischemia fell close to those derived from CMR_{O_2} . However, results indicate a gradual fall in $\sim P$ utilization with time of ischemia and, after 20 s, the value was significantly less than the 5 s figure. In contrast, phenobarbital anesthesia gave identical values for 10 and 20 s, somewhat lower than that expected from CMR_{O_2} .

The results of Table III corroborate the findings of Lowry *et al.* (1964) that calculated $\sim P$ utilization falls off with time. In their study a linear rate of $\sim P$ use was obtained in the first 15 sec. However, our data indicate that 'maximal' values in N_2O anesthesia are obtained only during the first 5 s. In contrast, identical values are obtained after 10 and 20 s in animals anesthetized with phenobarbital. Following the initial report of Lowry *et al.* (1964) most subsequent studies have been carried out with ischemic periods of 15 s or less (Gatfield *et al.* 1966, Folbergrova *et al.* 1970, Brunner *et al.* 1971, Ferrendelli and O'Rand 1973). Probably the $\sim P$ values so obtained are too low because the ischemic period has been extended into the nonlinear part of the curve. An exception is provided by the work of Swaab and Boer (1972) who used the period 2–8 s and obtained a $\sim P$ utilization ($\mu\text{mol g}^{-1} \text{ min}^{-1}$) similar to the initial one reported here.

Even if the period of ischemia is kept short and well-defined by using a rapid free-flow technique, the values of pre-ischemic energy utilization rate obtained by the closed system method must be regarded with some caution. First, the method used for inducing ischemia may increase metabolic rate above normal (see Nilsson and Nordström 1977). Second, if metabolic rate is normal or increased, very short ischemic periods must be used (see above). Third, since values close to those derived by a quantitative measurement of CMR_{O_2} are obtained only when a very short (5 s) period of ischemia is used, the possible error introduced by the oxygen content of the blood and tissue must be considered. The oxygen content of cerebral blood in the rat can be assumed to be $6.7 \mu\text{mol ml}^{-1}$ and it may be assumed that each g of cortical tissue contains approximately 0.01 ml of capillary blood. The solubility coefficient for O_2 in brain tissue is $2.2 \times 10^{-4} \text{ l g}^{-1} \text{ atm}^{-1}$ (T

Effect of sympathetic stimulation on the blood brain barrier dysfunction induced by amphetamine and by epileptic seizures

By

BARNÖD B. JOHANSSON and STAFFAN LUND

Received 1 March 1978

Abstract

JOHANSSON, B. B. and S. LUND: *Effect of sympathetic stimulation on the blood brain barrier dysfunction induced by amphetamine and by epileptic seizures*. Acta physiol. scand. 1978. 104: 281-286.

Stimulation of the sympathetic nerves to the brain is known to make the resistance vessels able to withstand higher blood pressure, to prevent blood-brain barrier (BBB) dysfunction and overperfusion in hypertension. When hypertension occurs concomitantly with metabolic vasodilatation, e.g. during epileptic seizures and after amphetamine-administration, protein leakage in the brain is more pronounced in hypertension per se. Unilateral stimulation of the cervical sympathetic chain during the administration of amphetamine or bicuculline—the latter GABA-receptor blocking substance that induces epileptic activity—attenuated the leakage of Evans blue-albumin and 125 I-HSA into the brain. Our results thus indicate an epileptic effect of sympathetic stimulation also when hypertension is combined with metabolically induced vasodilatation. The sympathetic nerves may constrict both extracerebral arteries and intracerebral resistance vessels. Unexpectedly the effect on the BBB of unilateral stimulation was to a great extent blocked under the present experimental conditions.

Key words: Sympathetic stimulation, blood-brain barrier, acute hypertension, amphetamine, bicuculline, epileptic seizures.

In acute hypertension, the mechanical stress on the cerebral vessels can increase the cerebrovascular permeability to protein (references see Johansson 1976). A blood pressure elevation and a concomitant pronounced cerebral vasodilatation are induced by bicuculline (Lekström and Nilsson 1976)—a drug that provokes epileptic seizures—and by amphetamine (Carlsson, Hägerdal and Siesjö 1975; McCulloch and Harper 1977). Vasodilatation increases the vessel wall tension, and the protein leakage in the brain in these two models is more extensive than that seen in, e.g. angiotensin-induced hypertension. Very characteristic and reproducible patterns of protein extravasation occur (Johansson and Nilsson 1977; Carlsson and Johansson 1978). As the BBB dysfunction can be prevented by various methods but abolishing the increase of blood pressure both in experimental seizures and after the

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Effect of sympathetic stimulation on the blood brain barrier dysfunction induced by amphetamine and by epileptic seizures

By

BARRO B. JOHANSSON and STAFFAN LUND

Received 1 March 1978

Abstract

JOHANSSON, B. B. and S. LUND. *Effect of sympathetic stimulation on the blood brain barrier dysfunction induced by amphetamine and by epileptic seizures.* Acta physiol. scand. 1978. 104. 281-286.

Stimulation of the sympathetic nerves to the brain is known to increase the resistance vessels able to withstand higher blood pressure, to prevent blood-brain barrier (BBB) dysfunction and overperfusion in hypertension. When hypertension occurs concomitantly with metabolic modulation of resting brain vessels and after amphetamine-administration, protein leakage in the brain is more pronounced in hypertension *per se*. Unilateral stimulation of the cervical sympathetic chain during the administration of amphetamine or bicuculline—the latter a GABA-receptor blocking substance that induces epileptic fits—increased the leakage of Evans blue-dye and ^{51}Cr -EDTA into the brain. Our results thus indicate a facilitative effect of sympathetic stimulation also when hypertension is combined with metabolically and vasodilatoric. The sympathetic nerves they control both extracerebral arteries and intracerebral blood vessels. Unexpectedly the effect on the BBB of unilateral stimulation is to great extent bilateral under the present experimental conditions.

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I. Mean arterial pressure (MAP) before and after the increase of blood pressure (induced by amphetamine or bicuculline). PaCO_2 and pH before the sympathetic stimulation and pressure increase. Mean values \pm SE. PaO_2 was >13.3 kPa (100 mmHg) in all rats.

Experimental groups		MAP before drug injection mmHg	MAP after	PaCO_2 kPa	pH
amphetamine, control	5	138 ± 3	186 ± 6	4.8 ± 0.1	7.37 ± 0.01
amphetamine, stimulation	5	139 ± 4	189 ± 9	4.7 ± 0.2	7.37 ± 0.01
bicuculline, control	7	144 ± 3	190 ± 3	4.7 ± 0.1	7.37 ± 0.01
bicuculline, stimulation	6	139 ± 2	192 ± 5	4.7 ± 0.1	7.36 ± 0.01

Arteries were taken for stimulation counting. Amphetamine induces leakage in cortical areas, predominantly in the anterior parts (Carlsson and Johansson 1978). The extravasation after bicuculline is more spread but is usually most pronounced in the thalamus (Johansson and Nilsson 1977). Therefore, in amphetamine groups the leakage control was determined in the cerebral cortex. In the bicuculline groups leakage was also estimated in the thalamus and the cerebellum. Statistical differences are tested with Wilcoxon test and Fisher's test.

Results

Rats given EB only 5 rats received amphetamine and 4 bicuculline during sympathetic stimulation. MAP exceeded 175 mmHg in all rats and at this pressure level protein leakage occurred in the brain in all normotensive rats in earlier series with amphetamine or bicuculline.

In the amphetamine group 3 brains did not show any EBA leakage: a faint blue discoloration occurred in the other two brains (slightly more in the left, non-stimulated, hemisphere in one of these). In the bicuculline group some EBA extravasation took place at least in the thalamus in all rats, but the leakage was less than is usually seen after a comparable blood pressure increase. A slight side-to-side difference was seen in one rat only (left > right).

Rats given both ^{125}I HSA and EB Table I presents MAP before and after the pressure increase induced by amphetamine or bicuculline in the various groups. *Amphetamine* In control rats, EBA extravasation was moderate in 3 and marked in 2 brains. Two of the brains from rats subjected to sympathetic stimulation did not show any EBA leakage in

Table II. ^{125}I HSA leakage in the brain after an amphetamine-induced blood pressure increase in rats subjected to stimulation of the right cervical sympathetic chain and in non-stimulated controls. The tracer content is expressed as $100 \cdot (\text{count min}^{-1} \cdot \text{mg}^{-1} \text{ brain tissue}) / (\text{count min}^{-1} \cdot \text{mg}^{-1} \text{ blood})$. Mean \pm SE.

Experimental groups	Right cerebral cortex	Left cerebral cortex
Control (n 5)	0.38 ± 0.11	0.37 ± 0.08
Stimulated (n 5)	$0.12 \pm 0.03^*$	0.12 ± 0.05

* $P < 0.05$ for significant differences from the controls.

administration of amphetamine these models can be used to study the effect of local intraluminal pressure on the BBB. As regards the cerebral metabolism there are important differences. Thus, the cerebral oxygen consumption is not increased in acute hypertension *per se* whereas the metabolic rate is greatly enhanced in bicuculline-induced ascots (Edrums and Nilsson 1976) and is increased also after the administration of amphetamine (Carlsson, Hägerdal and Siesjö 1975; McCulloch and Harper 1977).

The BBB studies mentioned so far have been performed on anesthetized animals. An angiotensin-induced hypertension can induce BBB dysfunction also in conscious rats (Johansson and Henning 1976). However, in a recent comparative study it was found that awake rats developed less BBB dysfunction than anesthetized rats when they were exposed to an abrupt rise in blood pressure (Johansson 1978). This was particularly evident when the pressure increase was induced by amphetamine or by bicuculline.

Bill and Linder (1976) have demonstrated that stimulation of the cervical sympathetic chain during acute hypertension prevents the blood flow increase and break-down of the BBB on the stimulated side. Studies by Edvinsson, Owman and Siesjö (1976) and MacLennan *et al.* (1977) have confirmed that a shift in the upper limit of the autoregulation towards higher absolute levels of arterial pressure occurs during sympathetic stimulation. The experiments suggest that one possible explanation to the difference between conscious and anesthetized animals to pressure-induced BBB dysfunction could be a difference in sympathetic tone. The present study was performed to see if unilateral stimulation of the sympathetic cervical chain could attenuate or prevent the BBB dysfunction when the blood pressure increase was combined with a metabolic vasodilatation.

Material and methods

Male Sprague Dawley rats (350–400 g) were used. Anaesthesia was induced with diethyl ether. The air tracheotomized, immobilized with sodiumthionam chloride and artificially ventilated. At 30°C and 70% N_2O . A femoral artery was cannulated for anaerobic sampling of blood and for recording of the mean arterial pressure (MAP), a femoral vein was cannulated for intra-venous (i.v.) injection of drugs and tracers. PaO_2 , PaCO_2 and pH were determined and only normocapnic rats with $\text{PaO}_2 > 100$ mmHg were included in the study. Evans blue (EB) in saline (1 ml kg^{-1}), which has no blood vessel permeability, was given i.v. before the injection of bicuculline or amphetamine to enable visual inspection of protein extravasation. The body temperature was maintained at approximately 37°C.

The right cervical sympathetic chain was isolated and sectioned approximately 1 cm below the superior sympathetic ganglion. The cranial end of the nerve was mounted on bipolar silver electrodes with the proximal electrode being cathode. The nerve and the electrodes were further surrounded by liquid paraffin to prevent drying of the nerve. Square wave pulses of 1 ms duration at 10 Hz were used. The threshold voltage was determined by observing the dilatation of the pupil and the appearance of exophthalmos on the homolateral side and 10 times this voltage was used for stimulation (maximum voltage used 1 V). The vagus nerve was usually left intact (in two experiments the nerve was sectioned for technical reasons). 1- α -amphetamine sulphate (2.5 mg kg^{-1}) or bicuculline (1.2 mg kg^{-1}) was injected i.v. 30 s after the beginning of the stimulation, which continued until the animal was killed by i.v. pentobarbital 3 minutes later. The extravasation of Evans blue-albumin (EBA) was registered and compared in the two hemispheres.

Because of the preliminary results from these series, an apparent bilateral effect of unilateral stimulation (see results), the experimental design was changed as follows. ^{125}I -serum albumin (^{125}I ISA) is given in addition to EB to allow a detailed comparison of the extravasation in the two hemispheres and in stimulated and non-stimulated animals. Amphetamine or bicuculline was injected either during sympathetic stimulation or to non-stimulated controls. Three minutes later the brains were briefly perfused *in situ* with saline (30 seconds) to remove tracer-containing blood from the cerebral vessels. Predetermined areas of

of blood pressure. Bilateral changes of cortical blood flow and of the diameter of pial vessels were also observed in some normotensive cats by Kobayashi, Waltz and Rhoton (1971). The extensive literature on the effect—or lack of effect—on the CBF by sympathetic stimulation in normotensive animals has recently been reviewed by Edvinsson and MacKenzie (1976) and will not be further discussed in the present paper.

The sympathetic innervation of cerebral vessels is basically unilateral (see Edvinsson 1975). In the rat, the two anterior cerebral arteries unite to form an unpaired anterior cerebral trunk (Zeman and Innes 1963). Thus, constriction of one of the supplying arteries could affect the blood flow also to the contralateral side and might help to explain the effect of sympathetic stimulation in the amphetamine model, in which the most severe extravasation usually takes place in the anterior part of the brain (Carlsson and Johansson 1978). However, this fact alone does not satisfactorily explain the functional bilateral protection of lateral stimulation found in the present series and by MacKenzie *et al.* (1977).

The difference between our results and those of Bill and Linder (1976) might possibly reflect species difference. Some differences in the techniques applied may also have played a role. Bill and Linder cut the vagus nerve on the stimulated side and on the contralateral side. Both the sympathetic trunk and the vagus were sectioned, a procedure which might have constituted a side difference. In the present study these nerves were left intact.

The vasodilatation seen in acute hypertension is mechanically induced whereas the vasodilatation induced by amphetamine is thought to be—at least in part—secondary to a catecholamine-induced increase of cerebral metabolism (Carlsson, Hågerdal and Sienjö 1975; Collochio and Harper 1977; Carlsson and Johansson 1978). There is a functional coupling between the increased metabolism and blood flow in experimental seizures but the trigger mechanism behind the vasodilatation is currently under debate and is probably complex (Astrup *et al.* 1976). It might seem surprising that sympathetic stimulation can influence metabolically induced vasodilatation. The effect of sympathetic stimulation is not necessarily an evasive effect on the intracerebral vessels. It has been suggested that the effect of sympathetic stimulation is primarily an effect on the carotid artery and its larger extracranial branches. Constriction of these arteries would normally lead to dilatation of vessels distal to the constriction because of autoregulation of the cerebral blood flow. However, in conditions with maximally dilated intracerebral vessels it would decrease CBF. This hypothesis has been proposed to explain the reduction in flow observed in hypercapnic animals during sympathetic stimulation (James, Millar and Purves 1969; Kobayashi, Waltz and Rhoton 1971; Harper *et al.* 1977). The greatest reduction in flow was seen at a high PaCO_2 when the initial blood flow was the highest.

In conclusion, our results indicate that the prophylactic effect of sympathetic stimulation on the BBB in acute hypertension—first demonstrated by Bill and Linder (1976)—is at hand when hypertension is combined with metabolically induced vasodilatation. It is not clear whether the sympathetic nerves exert an influence predominantly on the intracerebral or extracerebral vessels or if it is a combined effect on both.

Excellent technical assistance was given by Barbro Eriksson and Ulla Kante. The study was supported by grants from the Swedish Medical Research Council (project No. 81X-4968) and from the Swedish National Association against Heart and Chest Diseases.

TABLE III Extravasation of ^{125}I HSA in the brain after the administration of bicuculline to rats with concomitant stimulation of the right cervical sympathetic chain and in non-stimulated rats. The tracer content is expressed as 100 (counts/min mg^{-1} brain tissue)/(counts/min mg^{-1} blood). Mean \pm SE.

Experimental groups	Cerebral cortex		Thalamus		Cerebellum
	right	left	right	left	
Control (n=7)	0.59 \pm 0.17	0.56 \pm 0.16	0.81 \pm 0.29	0.85 \pm 0.32	0.55 \pm 0.05
Stimulated (n=6)	0.16 \pm 0.02	0.19 \pm 0.03	0.51 \pm 0.05	0.54 \pm 0.05	0.52 \pm 0.05

$p < 0.01$ for significant differences from the controls.

two brains a faint and in one brain a moderate blue staining was seen. ^{125}I HSA content in cortical areas from the stimulated and non-stimulated side is presented in Table II in agreement with the visual estimation of EBA extravasation, no difference was observed between the right and left hemisphere (In comparison it should be mentioned that the corresponding tracer content in normal rat brain, i.e. from rats not subjected to hypertension, is 0.02–0.04; see Carlsson and Johansson 1978). *Bicuculline* In the controls the pattern and degree of EBA extravasation was in agreement with earlier series, i.e. protein leakage occurred in many regions including the cerebral cortex but was most pronounced in the thalamus. Some blue staining was present in the thalamus also in the stimulated rats (left side slightly more than right in two brains) (the EBA leakage in the cortical areas was slight or absent. The fact that bicuculline increased MAP more in the stimulated group than in the controls (see Table I) would *per se* be expected to result in a higher degree of protein extravasation and explains why the protein leakage from the cerebellar vessels, which has a sparse sympathetic innervation (Edvinsson 1975) was more pronounced in the stimulated group than in the controls (see Table III). The effect of sympathetic stimulation was stronger on cortical resistance vessels than on vessels in the thalamus. The difference in protein leakage in the thalamus between stimulated rats and controls was not significant.

Discussion

The bilateral preventive effect on the BBB function produced by a unilateral sympathetic stimulation was unexpected. A slight difference between the stimulated and non-stimulated side was noticed in a few rats. The only marked side-to-side difference we have observed was in a rat given amphetamine which was excluded from the series presented here because it was hypercapnic (cf. Johansson and Nilsson 1977). Bill and Linder (1976) reported a marked side difference as regards both CBF and BBB function between the stimulated and non-stimulated side in their experiments on acute hypertension in cats. On the other hand, MacKenzie *et al.* (1977) have found a bilateral effect on the CBF by unilateral stimulation in acutely hypertensive baboons. They found no consistent side-to-side difference at any

Estrogen induced inhibition of ³H-noradrenaline release in the uterus and portal vein of the rat

By

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Abstract

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The influence of estrogen treatment on ³H-noradrenaline release, induced by potassium or calcium, was studied in isolated preparations of the uterus and the portal vein of the rat. Uterine strips of oophorectomized rats responded with contraction followed by transient relaxation when immersed in solution containing 127 mM potassium. The transient relaxation was accompanied by an increased rate of ³H-noradrenaline release. The transient relaxation was accompanied by an increased rate of ³H-noradrenaline release. When the uterus strips were bathed in calcium-free potassium solution, addition of calcium (3 mM) evoked an increased outflow of ³H-noradrenaline coinciding with relaxation phase of contractile response to calcium. After estrogen treatment of the rats the pattern of response was altered. Uterine strips responded to potassium and calcium with sustained contractions and with relatively little (10^{-4} M) increase of ³H-noradrenaline release. Noradrenaline (10^{-6} M) had no effect on the uptake or release of ³H-noradrenaline in either non-estrogenized or estrogen treated uterus. Calculations, based on the amounts of ³H-noradrenaline released by potassium and on the inhibitory effect of desipramine (10^{-6} M) on uterine uptake of noradrenaline, suggested that similar amounts of transmitter were accumulated in the adrenergic nerves of both non-estrogenized and estrogen treated preparations. Tyramine (10^{-4} M) had a weaker stimulatory effect on ³H-noradrenaline release than potassium, and the tyramine induced release was not inhibited by estrogen treatment. Strips of the rat portal vein responded to potassium with an increased rate of ³H-noradrenaline release. As in the uterus, estrogen treatment reduced the release of ³H-noradrenaline in response to potassium. The adrenergic effect of tyramine was possibly due to reduced entry of calcium ions into the nerve terminal.

It has previously been presented that smooth muscle contractions, induced by potassium or calcium, are under certain conditions modified by the release of intramural noradrenaline (Bengtsson 1977 a, 1978). Thus the contractions of the rat uterus exhibited a transient relaxation that did not appear in a reserpinized preparation or after β -adrenoceptor blockade. The contractile response of the rat portal vein, on the other hand, was enhanced by the release of endogenous noradrenaline. An additional finding was that estrogen treatment completely (uterus) or partially (portal vein) inhibited the contractile effects of intramural noradrenaline. This sympatholytic effect of estrogen could not be accounted for at least not on a quantitative basis, by decreased sensitivity of the adrenoceptors, suggesting that estrogen might reduce the amount of noradrenaline released.

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However, estrogenized strips were not divided longitudinally and no ^3H -sucrose was added to the incubation medium. Each specimen was transferred through a series of 4 tubes containing tracer-free

The first 3 tubes of each series contained Na-Krebs. The last tube contained K-Krebs in 3 of the and tyrosine in Na-Krebs in the 4th one. The time schedule for bathing the tissues was 10–15 min.

Drugs used, except tyrosine, were present in the solutions from the beginning of the equilibration to the end of the experiment.

of run. The experimental design was essentially the same as that described for the uterus under

of *H-noradrenaline* and ^3H -sucrose. The preparations were weighed before mounting on gel tubes and dissolved in a solvent as described by Batra and Bengtsson (1976), at the end of the run. A toluene-Triton-X-100 solution was used as scintillation fluid (Patterson and Grease 1965). Radioactivity was measured in liquid scintillation counters (Packard Tri-Carb or Nuclear Chicago 300). Quenching was corrected for by using the external standard method.

calculus. The contractile responses were read directly from the original recordings whereas the of the isotope measurements have been treated as follows. In all experiments the ^3H -noradrenaline content of the tissue at any given time was calculated by adding the total amount of ^3H -noradrenaline released in the time to that remaining in the tissue at the end of the experiment. In the A- and B-series and in the experiments, effect of ^3H -noradrenaline is expressed as rate coefficient (r) in min^{-1} .

$$\frac{A}{\Delta t \cdot Q}$$

A represents the radioactivity loss in the time interval Δt , and Q is the content of ^3H -noradrenaline in the tissue at the midpoint of the time interval Δt . In the C-series the rate of ^3H -noradrenaline release is used as min^{-1} ($A/\Delta t$). Further details on calculations are given under Results.

Statistical significance was determined by the use of Student's t -test.

Media

Preparations were bathed in Krebs solution (Na-Krebs) of the following composition (mM): NaCl 119.46, NaHCO_3 20, CaCl_2 1.5, NaH_2PO_4 1.2, MgCl_2 1.2, glucose 11, pH 7.4. When the effects of de-ionized water were studied as isotonic potassium solution (K-Krebs) of the following composition was used (mM): KCl 127, NaHCO_3 20, CaCl_2 1.5, NaH_2PO_4 1.2, MgCl_2 1.2, glucose 11, pH 7.4. In the calcium-potassium solution, CaCl_2 was omitted and EDTA (0.01 mM) was added. When effects of calcium were studied, a concentrated solution of CaCl_2 was added to the calcium-free potassium solution (final 3 mM). EDTA ($3 \cdot 10^{-4}$ M) and sodium ascorbate ($5 \cdot 10^{-4}$ M) were added to the media to prevent oxidation of noradrenaline. The solutions were equilibrated with mixtures of 5% CO_2 and 95% O_2 and at 37°C.

Drugs and reagents

Apomorphine (Ciba), norepinephrine (Sigma) and tyrosine (BDH). DL-noradrenaline-7- ^3H (8.9 Ci/mole) and sucrose- ^3H (5 mCi/mole) (New England Nuclear Corporation).

Results

Uterus

Contractile responses and release of *H-noradrenaline*

In Na-Krebs solution the uterus contracted spontaneously. When this medium was replaced by K-Krebs containing 1.5 mM calcium, a brief myometrial contraction was followed by a pronounced relaxation (Fig. 1). The muscular tension transiently increased when the solution containing K-Krebs was replaced by calcium-free potassium solution. Addition of 1.5 mM calcium to the isotonic potassium solution caused a phasic increase in tension fol-

It has been the aim of the present investigation to further characterize the release of intramural noradrenaline during potassium and calcium induced contractions of uterine muscle. To this end the time course of potassium and calcium induced release of intramural noradrenaline has been studied and correlated with the mechanical events in the uterine portal vein of the rat. It has also been investigated whether the sympatholytic effect of oestrogen is mediated through inhibition of noradrenaline release from the adrenergic nerve or if increased extraneuronal binding prevents the noradrenaline from acting on the adrenergic receptors. A preliminary report on this investigation has been given previously (Bengtsson 1977 b).

Methods

Animals

46 virgin female rats of the Wistar strain, weighing 150–200 g were used. All animals were oophorectomized and half the number were given 0.5 mg of polyestradiol phosphate (Lm, immediately after operation). The dose (related to body weight) of polyestradiol phosphate is about double that used clinically to treat menopausal symptoms. There were close similarities between oestrogen treated and estrus rats and non-oestrogenized and diestrus rats, respectively as to macroscopical appearance of the uterus. Corresponding results were found when calcium contractions were evoked in the depolarized uterus (Bengtsson 1979). Oestrogen treated rats were killed on day 4 and the rest of the animals (non-oestrogenized) were used on day 12 after operation. Myometrial contractions evoked by potassium or calcium displayed the same pattern as in day 4 as on day 7.

Preparations and experimental procedures

Either the uterus or the portal vein were isolated and placed in Na Krebs solution after which they were split open (the uterus at the mesenteric border) longitudinally.

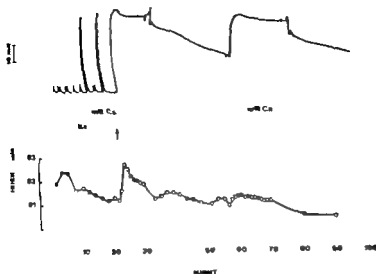
Uterus Procedure A About 20 mm of the middle part of one of the uterine horns was used. The tissue was mounted longitudinally in a 2 ml jacketed organ bath containing Na Krebs and subjected to a tension of 10 mN which within 10 min subsided to a steady level at 1–2 mN. After an accommodation period of 30 min the tissue was incubated with H -noradrenaline ($4 \cdot 10^{-6}$ M) and 3C -sucrose ($3 \cdot 10^{-3}$ M) in Na Krebs.

3C -sucrose was used as an extracellular marker in order to investigate whether tracer substance could be squeezed out of the tissue as a result of the contraction. When the tissue was contracted by potassium a small increase of the rate constant of 3C -sucrose efflux could be seen in some experiments. However, this increase was less than 1/10 of that seen for H -noradrenaline on stimulation with potassium.

After 60 min of tracer uptake the uterus and the bath were rinsed three times with non-radioactive Na Krebs (total time for rinsing 15 s). Then the muscle chamber was continuously perfused at detector rates (0.5–2 ml/min) with isotope-free solution which were collected in 2 ml samples through a tube at the top of the bath. All gas was obtained by bubbling the fluid with 95% O_2 and 5% CO_2 from the bottom of the bath. The contractile activity of the muscles was recorded isometrically by a tension transducer (Grass FTO3-C) connected to an ink-writer (Beckman R 611).

Procedure B About 70 mm of the middle part of one uterine horn was used. Oestrogenized strips were divided longitudinally in order to reduce the weight difference between untreated and oestrogenized strips. After this modification the weight of the oestrogenized preparations varied between 46 and 65 mg whereas the weight of untreated tissue was 43–48 mg. The strips were mounted on tubes of stainless steel, according to their natural length, and placed in Na Krebs for an equilibration period of 30 min. The fluid was removed by bubbling with 95% O_2 and 5% CO_2 through the steel tubes. After equilibration the suspended strips were placed in plastic tubes containing 3 ml Na Krebs with $4 \cdot 10^{-6}$ M H -noradrenaline and $3 \cdot 10^{-3}$ M 3C -sucrose. The incubation with isotopes lasted for 60 min after which tissues and steel tubes were rinsed 3 times in 100 ml non-radioactive Na Krebs (total rinsing-time 15 s). The preparations were then transferred through a series of plastic tubes each containing 3 ml of tracer-free solution. The tissue was removed for 10 min in each tube. Isotopes recovered in the bathing solution of these tubes were measured by counting radioactivity in 2 ml aliquots.

Procedure C Both uterine horns were used. They were divided by transverse section into 2 pieces of about equal length, giving 4 strips from each uterus. These strips were essentially treated as described



Mechanical activity (upper trace) and rate coefficient of ^3H -noradrenaline efflux (lower trace) in isolated rat uterus. Perfusing media as indicated between the traces. Na^+ - Na-Krebs, K - K Krebs, minutes shift from Na to K.

used the contracture tension in estrogen treated uterus. No significant spontaneous action was observed in this case, in line with the finding that calcium did not cause any rise of the ^3H -noradrenaline release.

Quantitative analysis of the release of ^3H -noradrenaline induced by potassium and calcium

Results shown in Fig. 1 and 2 indicated that both potassium- and calcium-induced noradrenaline release in the rat uterus was impaired by estrogen treatment. In order to estimate the ^3H -noradrenaline release, the latter was studied in tissues (7 untreated and estrogenized rats) which were exposed to Na-Krebs, K Krebs and increased calcium concentration in the same order as in the experiments described under A. The mean rate coefficient of noradrenaline efflux into these solutions is shown in Fig. 3. The rate constant of ^3H -noradrenaline release in K-Krebs containing 1.5 mM calcium (third column) was $0.038 \pm 0.001/\text{min}$ in the untreated and $0.019 \pm 0.002/\text{min}$ in the estrogenized tissue. When the noradrenaline release was stimulated by 3 mM calcium (last fraction) the mean rate coefficient of efflux was $0.039 \pm 0.001/\text{min}$ in non-estrogenized uterus whereas the corresponding value for estrogenized preparations was only $0.014 \pm 0.001/\text{min}$. In consequence there remained a larger fraction of ^3H -noradrenaline in the estrogenized (0.246 ± 0.018) than in the untreated tissue (0.007) after the washout period.

Effects of desipramine, normetanephrine and tyramine on uptake and release of ^3H -noradrenaline

The data presented in the previous sections suggest that the release of ^3H -noradrenaline from adrenergic nerve endings was reduced by estrogen treatment. However the reduction

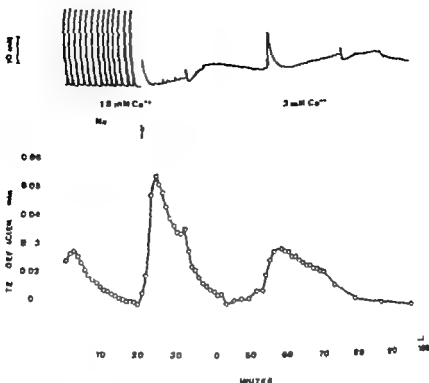


Fig. 1 Mechanical activity (upper trace) and rate coefficient of ³H-noradrenaline efflux (lower trace) from non-estrogenized rat uterus. Perfusing media as indicated between the traces. Na = Na-Krebs, K = K-Krebs, arrow indicates shift from Na to K.

lowed by a slowly developing contracture. When calcium was again omitted from the bath the preparation relaxed slowly.

In the lower part of Fig. 1 the simultaneous efflux of ³H-noradrenaline has been plotted as rate coefficient against time. The rate coefficient declined from an initial value of approximately 0.025/min to 0.009/min within the first 20 min of the washout period. This had no visible effect on the contraction. When the bathing medium was changed from Na-Krebs to K-Krebs the rate coefficient of ³H-noradrenaline efflux increased and reached a maximum of 0.054/min which coincided in time with the spontaneous relaxation phase of the potassium contracture. The efflux rate then declined and this was paralleled by the partial restoration of the contractile tension. After omission of calcium from the extracellular medium the rate constant of noradrenaline efflux continued to decrease and a final value of about 0.01/min was reached. Addition of calcium (3 mM) to the potassium solution evoked an increased outflow of ³H-noradrenaline coinciding with the relaxation phase of the calcium induced contracture.

Estrogen treatment of the rats modified the contractile responses of the isolated uterus (Fig. 2, upper tracing). The spontaneous activity in Na-Krebs was generally infrequent and irregular in the estrogenized myometrium. In this preparation K-Krebs produced a sustained contracture and it can be seen that there was a relatively small release of ³H-noradrenaline in this case. Omission of Ca from the bathing fluid caused a decline of the contractile tension, but had very little effect on the ³H-noradrenaline release. Addition of calcium

Effect of drugs on uptake and release of ³H-noradrenaline in rat uterus

Treatment	Weight of uterus strips (mg)	³ H-noradrenaline in uterine strips before release (pmol)	Releasing agent	H-noradrenaline released by K or tyramine (pmol/mg)
Non-estrogenized (n = 6)				
Control	28.5 ± 1.4	32.3 ± 3.1	K	4.00 ± 0.58
Desipramine 10 ⁻⁶ M	28.3 ± 2.2	9.1 ± 1.3	K	0.21 ± 0.14
Normetanephrine 5 · 10 ⁻⁶ M	27.8 ± 2.1	51.3 ± 3.7	K	3.76 ± 0.20
Normetanephrine 5 · 10 ⁻⁶ M	26.2 ± 2.6	37.7 ± 3.1	Tyramine 10 ⁻⁴ M	0.31 ± 0.07
Estrogen treated (n = 6)				
Control	86.5 ± 10.4	59.9 ± 2.5	K	1.49 ± 0.28 ^b
Desipramine 10 ⁻⁶ M	82.2 ± 7.3	31.1 ± 2.9	K	0.42 ± 0.12
Normetanephrine 5 · 10 ⁻⁶ M	80.8 ± 7.7	58.3 ± 4.0	K	2.06 ± 0.33 ^b
Normetanephrine 5 · 10 ⁻⁶ M	81.0 ± 7.9	51.8 ± 3.9	Tyramine 10 ⁻⁴ M	0.47 ± 0.10

uterine strips were from the cervical end in the control and normetanephrine-K series and from the ovarian end in the desipramine and normetanephrine-tyramine series.

from non-estrogenized *p* < 0.01.

was a considerable weight difference between the groups, the estrogenized preparations weighing 3-4 times as much as the untreated ones.

tissues were exposed to potassium or tyramine after 21 min of efflux in Na-Krebs. In time most of the ³H-noradrenaline in the extracellular fluid had left the tissue according to expts. in which ¹⁴C-sucrose was used as a marker for extracellular binding (see methods). The calculated amounts of ³H-noradrenaline existing in the preparations immediately before they were subjected to potassium or tyramine are given in Table 1. In these expts. the main question was whether a given number of adrenergic nerves in the uterus would respond with reduced transmitter release after estrogen treatment. For this purpose the tissue contents and the amounts of released ³H-noradrenaline have not been standardized with respect to tissue weight as this would produce seemingly small values in the estrogenized uterus (*cf.* above). The estrogen treated tissues contained somewhat larger amounts of tracer than the non-estrogenized preparations after immersion in the control medium. After inhibition of neuronal uptake by desipramine the amount of ³H-noradrenaline was reduced considerably in the non-estrogenized and to a lesser degree in the estrogenized uterine strips. Normetanephrine, on the other hand, did not influence the contents of ³H-noradrenaline in either non-estrogenized or estrogen treated tissue. The difference between the tracer contents in the two series of normetanephrine treated preparations (*cf.* expts 3 and 4 and 7 and 8, respectively) is probably explained by the fact that the cervical end of the rat uterus, used in the normetanephrine-K series, has more dense adrenergic innervation than the ovarian end (Adham and Schenk 1969; Hervonen *et al.* 1973), used in the normetanephrine-tyramine series.

Table 1 also shows the effects of potassium and tyramine on the rate of ³H-noradrenaline

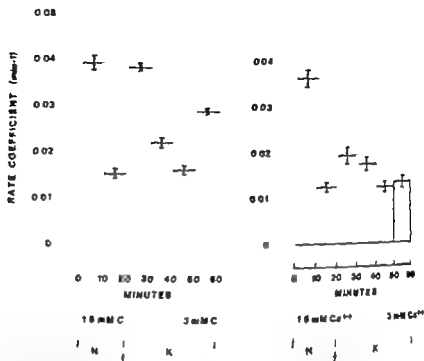
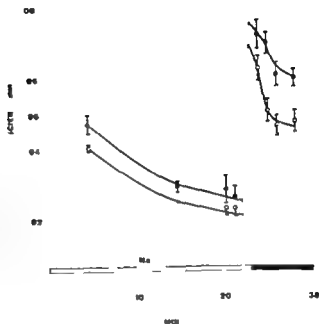


Fig. 3 Histograms showing rate constants of H-noradrenaline efflux from non-estrogenized (left) and estrogen-treated (right) rat uterus. The preparations were consecutively bathed for 10 min in media of composition indicated below the graphs. Na = Na-Krebs, K = K-Krebs, arrow indicates shift from Na to K. Values are mean \pm S.E. of 7 (non-estrogenized) and 6 (estrogen-treated) pts.

of the efflux coefficient in the estrogenized uterus might also be due to other mechanisms. There is a pronounced hypertrophy of uterine tissue without a concomitant increase in the amount of noradrenaline in the rat uterus under estrogen treatment (Oskarsson & Falck *et al.* 1974). Thus the possibility existed that the extraneuronal fraction of H-noradrenaline was much larger in the estrogen-treated uterus, and that a spuriously low rate of the potassium- or calcium-induced neuronal release therefore was obtained in this test. In order to examine this possibility the uptake and the release of H-noradrenaline were investigated in the presence of 1 desipramine which selectively blocks neuronal uptake (Titus and Spiegel 1961; Slag *et al.* 1963) and α -normetanephrine which has been shown to inhibit extraneuronal uptake (Borgen and Iversen 1965; Eisenfeld *et al.* 1967). As a means of clarifying the estrogenic effect on H-noradrenaline release was to induce release of noradrenaline with an agent different from potassium and calcium. For this purpose tyramine was chosen, as this substance has been shown to release noradrenaline by a process that is independent of calcium (Lindmar *et al.* 1967). The tissue uptake and release were followed in drug-free solution (control) and in solutions containing desipramine (10^{-5} M) or normetanephrine (5×10^{-6} M). There were 2 sets of solutions with normetanephrine, one for potassium-induced and one for tyramine-induced release of noradrenaline.

When preparing the uterine strips for this experiment, care was taken to divide each pair of uterine horns into 4 equal pieces. The mean weight of four sets of strips from untreated and estrogenized uterus, respectively, is shown in Table I. There was little difference between the weights of tissues within the groups of untreated and estrogenized preparations, whereas



Rate coefficient of ^3H -noradrenaline release in isolated rat uterus. Solid symbols: non-estrogenized. Open symbols: estrogen-treated. Bathing solutions as indicated below the graph. Na = NaCl, K = K-Krebs. Values are \pm S.E. of 7 experiments.

the values of A, Q and W obtained from Table I, the extraneuronal ^3H -noradrenaline retention was found to be 0.24 pmol/mg in the non-estrogenized and 0.25 pmol/mg in the estrogen treated uterus. These concentrations were multiplied by weight and subtracted from the total amounts of ^3H -noradrenaline in the tissue. The remaining amounts were considered as the neuronal ^3H -noradrenaline, were 45.4 pmol in non-estrogenized and 34.3 pmol in estrogen treated preparations, respectively. The calculations also suggested that the amount of ^3H -noradrenaline accumulated by the adrenergic nerves in the case of desipramine, was larger in the estrogen treated (10.6 pmol) than in the non-estrogenized (2.3 pmol) uterine strips.

The total amount of noradrenaline in the control preparations (considered as representing the whole uterus) would be approximately 130 pmol according to the values previously reported for the whole uterus including the cervix (Palck *et al.* 1974). Thus the estimated amounts of ^3H -noradrenaline, accumulated in the adrenergic nerves under control conditions (see above), represented about 30% of the endogenous transmitter.

Portal vein

non-estrogenized and estrogen treated strips of the portal vein weighed 5.5 ± 0.4 mg and 4.2 ± 0.4 mg, respectively. The contents of ^3H -noradrenaline in the strips immediately after they were exposed to K-Krebs were 2.49 ± 0.12 nmol/g in the non-estrogenized and 5.0 ± 0.12 nmol/g in the estrogen treated preparations. As the concentration of noradrenaline in the rat portal vein has been found to be 2.2 $\mu\text{g/g}$ (Häggendal *et al.* 1970), the accumulated ^3H -noradrenaline in the present experiments represented 20–25% of the endogenous transmitter.

release. The values were calculated by subtracting the efflux rate in the last fraction of Krebs from the rate of release during the subsequent 1 min immersion in K-Krebs. Tyramine-containing Na-Krebs. In both the control and the normetanephrine series potassium induced release of ^3H -noradrenaline was larger in non-estrogenized than in estrogen treated preparations. The desipramine treated strips released only small amounts of ^3H -noradrenaline when immersed in K-Krebs. Tyramine in Na-Krebs had a much less effect on ^3H -noradrenaline release than potassium. This release was not inhibited by estrogen treatment.

It was also of interest to estimate the amount of ^3H -noradrenaline that was present in adrenergic nerves when release was triggered by potassium. Provided that the desipramine induced block of neuronal uptake of noradrenaline was effective, the concentration of extraneuronal ^3H -noradrenaline would be given by the tracer concentration found in desipramine treated tissue. Accordingly the neuronal ^3H -noradrenaline should be derived from the difference between the total amount of tracer in the control and the amount of extraneuronal ^3H -noradrenaline. The nerve bound ^3H -noradrenaline estimated in this way was 43.1 pmol in the non-estrogenized and 27.2 pmol in the estrogen treated strips. These values suggest that estrogen treatment decreased the neuronal uptake under control conditions. However, the possibility exists that estrogen reduces the blocking effect of desipramine on the neuronal uptake of noradrenaline. This would give an underestimation of the nerve content of ^3H -noradrenaline in the estrogenized preparations.

An attempt was therefore made to differentiate between neuronal and extraneuronal ^3H -noradrenaline by calculations that were independent of a complete inhibition of neuronal uptake by desipramine. To perform these calculations the following assumptions were made: 1) The rate of potassium induced ^3H -noradrenaline efflux was proportional to the nerve content of tracer. This assumption is partly based on the previous finding that thymoleptic drugs had either no effect (Paton 1973) or only a slight inhibitory action (Sjöstrand *et al.* 1972) on ^3H -noradrenaline efflux when used in concentrations similar to that used for desipramine in the present study. 2) The concentration of extraneuronal ^3H -noradrenaline was equal in control and desipramine treated tissue. This is reasonable as the uterine contents of ^3H -noradrenaline were not affected by normetanephrine (see Table I), a more effective inhibitor of extraneuronal uptake than desipramine (Iversen 1967). 3) The weight of adrenergic nerves was negligible in relation to the weight of extraneuronal tissue in the uterus.

On the basis of these assumptions the following equation applies

$$\frac{A_0}{Q - B \cdot W} = \frac{A_d}{Q_d - B \cdot W_d}$$

A - rate of potassium induced ^3H -noradrenaline efflux

B - extraneuronal ^3H -noradrenaline concentration

■ - index of control tissue

d - index of desipramine treated tissue

Q - ^3H -noradrenaline in whole tissue

W - tissue weight

calcium by the addition of tyramine (Lindmar *et al.* 1967 Garcia *et al.* 1976). The study showed that the tyramine induced release of ³H-noradrenaline in estrogen preparations is undiminished. This further supports the idea that the estrogen inhibition of noradrenaline release is mediated through calcium dependent mechanism. The finding that tyramine induced release of ³H-noradrenaline was unaffected by this strongly suggests that the amount of ³H-noradrenaline, available for release in the terminals, is about the same in non-estrogenized and estrogen treated uterus. This is in agreement with the calculations shown under Results.

Rat portal vein responded like the rat uterus with an increased release of ³H-noradrenaline when immersed in the potassium-rich solution. This is consistent with a previous report on potassium contractures in the rat portal vein, suggesting that released intramural noradrenaline exerts a contractile effect which adds to the contracture produced by potassium itself (Bengtsson 1977 a). The previous findings also suggested that the adrenergic tone in the rat portal vein was reduced by estrogen treatment, probably through a reduction of noradrenaline release. This idea is supported by the present results, as estrogen treatment significantly reduced the potassium induced release of ³H-noradrenaline. It is probable that the basic mechanism for this reduction is the same as that already discussed for the uterus.

We thank Professor K. A. P. Edman for advice and helpful criticism during the course of this research, Professor K. E. Andersson and Dr B. Lyng for helpful discussions and criticism of the manuscript and Mrs L. Sjöman-Nilsson for skilful technical assistance.

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The rate coefficient of ^3H -noradrenaline efflux decreased from an initial value of $0.05/\text{min}$ to $0.02\text{--}0.03/\text{min}$ during the 23 min of washout in Na-Krebs (Fig. 4), and is larger in the non-estrogenized than in the estrogen treated preparations. This effect remained when the portal veins were transferred from Na to K Krebs. The rate coefficient of ^3H noradrenaline efflux increased to approximately $0.07/\text{min}$ during the first minute in potassium rich solution, after which the efflux rate showed a decline that is pronounced in the estrogen treated than in the non-estrogenized portal veins. The effect of potassium on ^3H noradrenaline efflux was calculated as the area under the curve for ^3H in K Krebs above the level of the rate coefficient in the last tube containing Na-Krebs. Values obtained in this way were 0.235 ± 0.13 in non-estrogenized and 0.192 ± 0.11 in estrogen treated portal veins ($p < 0.05$).

Discussion

Previous studies on potassium induced contractures in smooth muscle have shown contractile effects that have been attributed to the release of intramural noradrenaline (Kiv 1966, Gibson and Pollock 1973, Bengtsson 1977a). The present investigation has provided direct evidence for this idea by showing a close correlation between increased ^3H -noradrenaline efflux and relaxation in the non-estrogenized rat uterus.

The initial high output of ^3H -noradrenaline in the isotonic potassium solution disappeared rapidly in spite of continued stimulation with potassium. A similar finding has been reported previously (Garcia *et al* 1976). It appears from that study that the decline in output after prolonged stimulation with potassium is due to failure of transmitter mobilization rather than to exhaustion of releasable transmitter stores.

The potassium induced release of ^3H -noradrenaline was considerably reduced in uterine strips treated with estrogen. A smaller fraction of released ^3H -noradrenaline appears in the bathing medium if estrogen induced a more effective uptake by the neuronal tissue or a decreased diffusion rate through the extracellular space in the uterus. The fact that neither tissue content of tracer nor potassium induced efflux were altered by normetanephrine indicates that the extraneuronal uptake was very small under the experimental conditions used. Impaired diffusion is also an unlikely explanation of the estrogenic effect as the rate of noradrenaline flux into the uterine tissue is evidently not influenced by estrogen treatment. Thus depolarized uterine strips attain more than 90% maximum relaxation to a given dose of noradrenaline in 1 min, whether or not estrogen treated (unpublished observations).

In view of these results, it is reasonable to suggest that the inhibition of ^3H -noradrenaline release is exerted through mechanisms regulating the release of transmitter from the adrenergic nerve endings. Potassium induced release of noradrenaline is dependent on calcium in the extracellular medium (Kirpekar and Wakade 1968, Garcia *et al* 1976). Thus estrogen might inhibit transmitter release by reducing the entry of calcium ions into the nerve terminals. In support of this idea the present results show that the ability of calcium to release noradrenaline disappears after estrogen treatment (see Fig. 1, 2, 3).

It is of interest to note that noradrenaline release is produced in the absence of an

Effects of pressure on fast axoplasmic flow. An *in vitro* study in the vagus nerve of rabbits

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Abstract

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Using rapidly directed streams of fluid it is possible to compress sections of a nerve *in vitro* by known forces, thus interrupting the supply of oxygen and nutrients to the nerve. Motor neurons of the vagus nerve of rabbits labelled with ^3H -leucine are used at room temperature. When nerve is subjected to 10 mmHg pressure fast axoplasmic flow was not altered, but at 30 mmHg there was a slight but consistent decrease, which is even more marked at 60 mmHg and still more at 90 mmHg. The pressure induced block of axoplasmic flow was reversible: when 60 mmHg pressure was applied for as long as 4 h, then the nerve was compressed for 15 h there was reversibility in only 5 out of 6 cases.

Increased intraocular pressure blocks fast axoplasmic flow (FAF) at the level of the lamina propria in the optic nerve. This has been demonstrated unequivocally for both the anterograde FAF coming from the eye (Hansson 1973, Anderson and Hendrickson 1974) and the retrograde FAF—which enters the eye (Mitschler *et al.* 1977). It is not clear whether the pressure induced block of FAF in the optic nerve is mechanical or ischemic in origin. Since this is of crucial importance, it was decided to attack the problem using an *in vitro* system. The present study reports a technique for exerting pressure on the vagus nerve without interrupting the oxygen supply and presents some results using this technique.

Materials and methods

Animals. Albino rabbits (New Zealand White) of either sex weighing between 2.0 and 3.5 kg were anaesthetized with 7 ml/kg of 3% urethane solution, administered intraperitoneally or slowly intravenously. Any surgical anaesthesia had been attained the animals were placed under heating lamp to keep the body temperature around 37°C.

Labelling of the motor neurons of the vagus nerve. The method originally described by Mura (1963, see also Frouin 1974, for more detailed description) was used and is described here only briefly. 30 μl ^3H -leucine (4-5.5 Ci/mmol, specific activity as 60°C mesol, concentration 1 $\mu\text{Ci}/\mu\text{l}$) was applied to the floor of the fourth ventricle in the vicinity of the superior nucleus of the vagus. The application is done with micro-

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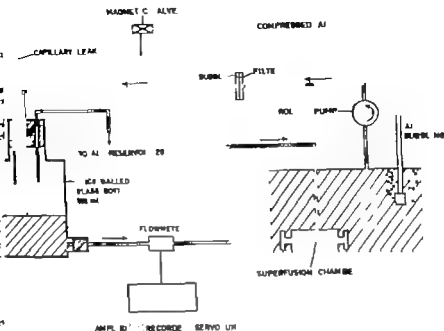


Fig. 1. Arrangement for superfusion of nerve with constant flow. For further explanation see text.

ing diameter of 1.1 and 1.3 mm. The position of the hole in the probe was indicated by black paint and was found that the hole was at the center of the gap when pressure was at its maximum. However the position was not sufficient to decide if this was true better than 0.1 mm. For reasons of symmetry it is assumed that the position of the gap is at the peak pressure, even if this is not proved.

Determination of radioactivity. The nerves were cut into 2-4 mm pieces and kept for 24 h in 10% trichloroacetic acid (TCA) at 4°C. Pilot studies showed that this treatment was sufficient to extract the TCA soluble fraction from the nerve tissue. The TCA soaked nerve pieces were either combusted in Packard 306 Tri-carb sample oxidizer or treated with Soloxene® to which scintillation liquid was added when digestion was complete. The radioactivity was determined in liquid scintillation counter and corrected for quenching and accuracy. The value for each nerve segment is expressed as percentage of the total amount in the whole nerve.

Results

Calibration of the superfusion chamber

The pressure distribution within the superfusion chamber is shown in Fig. 3. For clarity only two sets of conditions are shown. At given flow the gap size defines the shape of the curve. The curves were analysed with regard to the following three parameters: the maximum pressure value (in mmHg), the distance in mm between the 30% points on the limbs and the steepest slope of the limbs in mmHg/mm. In Fig. 4 the maximum pressures are plotted against different flows for different gap openings. It is apparent from this graph that the smaller the gap and the greater the flow rates, then the greater is the maximum pressure. Values between 30 and 150 mmHg could easily be achieved. While the slope also depends on the flow rate at

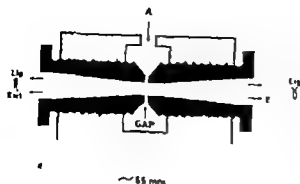


Fig. 1 Superfusion chamber. The two identical collinear screws, 27 mm long, are of plexiglass. Their central channel has a diameter of 2.5 mm, is cylindrical initially and expands conically to 3 mm. The screws are mounted within the chamber. Fluid at a constant rate enters at A and runs radially to the gap, comprises the nerve and is the channel along the nerve (to the nodulum).

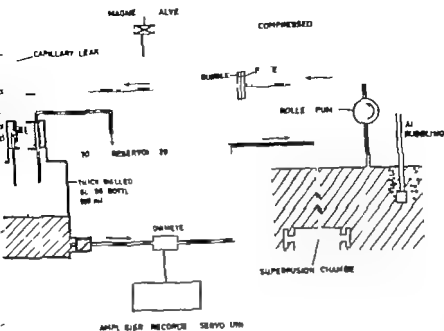
syringe (Aglia) at a rate of 1.2 μ l/min. After 3.5 h the animal was killed by either an air embolism or a lethal injection of a overdose of urethane. Then, without delay 50–70 mm of both cervical vagi was removed between two ligatures. The proximal ligature was applied just rostral to the ganglion nodulum. The removed nerves were then immediately transferred into cold aerated superfusion solution and freed from connective tissue under the microscope. Care was taken not to touch or squeeze the nerve.

The composition of the superfusion solution was: 1 000 ml 0.9% NaCl, 40 ml 1.15% KCl, 12.7 ml CaCl_2 , 10 ml 2.11% KH_2PO_4 , 10 ml 3.13% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 210 ml 0.1 M Na₂HPO₄ \cdot 2H₂O, 2.57 g/l glucose.

Localized compression of a nerve. The method was designed so that a small segment of a nerve could be compressed by an applied constant pressure without interrupting the supply of oxygen. The device is shown in Fig. 1. The nerve is placed in the longitudinal channel of the chamber. Aerated superfusion air enters the chamber at A at a constant and rapid rate and flows centrifugally through an adjustable gap where it impinges on the nerve and escapes axially along the nerve. A positive pressure is then applied in the radial direction around the whole perimeter of the nerve as it passes through the gap. The channel for the nerve runs through the long axis of two collinear plexiglass screws and the gap is formed between their ends. Gap size can be adjusted by turning the screws which can be locked by counterlocks. No attempt was made to calculate the complex fluid dynamics within the longitudinal channel. Instead the pressure distribution in the channel was measured by a probe (see below). The pressure profile was calibrated for different flow rates passing through the chamber for different gaps. Unfortunately it was technically impossible to record the pressure within the chamber continuously during an experiment. However by maintaining constant flow at a given gap size the shape of the pressure distribution could be kept constant. Fig. 2 shows the whole arrangement. A roller pump (Watson-Marlow) sucks the superfusion solution and air from the surface of an aerated bath—thus keeping the surface level of the bath constant. The fluid passes through a filter (consisting of a nylon net with mesh aperture of 15 μ m) and enters the thick-walled glass bottle in which the fluid is driven by air pressure and passes through a flowmeter into the superfusion chamber. The bath. The flowmeter consists of a constriction plexiglass tube and differential pressure transducer (Ranborn, type 267 B). The flowmeter is part of a servo system maintaining constant flow. If the flow decreases below the present value, a magnetic valve opens and air pressure is applied. This dampens out the pressure fluctuations the air space over the fluid in the bottle was connected to a 20 l expansion chamber. A very constant flow could thus be obtained for an extended period of time. Superfusion was usually maintained for 17 hrs at room temperature. At the end of the experiment the nerve was cut across in situ at the center of the gap with a scalpel, the blade thickness of which fitted the gap. The two separated parts were then divided further into 2–4 mm pieces and the radioactivity determined as described below.

Pressure probe. The probe used for determining the pressure distribution in the channel for the superfusion chamber consisted of a steel tube 150 mm long and 0.9 mm in outer diameter. A 0.1 mm hole was drilled approximately midway in the side wall. One end was plugged while the other was fitted with a plexiglass tube connected to a transducer (Ranborn, type 267 B). At the beginning of a measurement the probe, plexiglass tube and transducer were connected to a reservoir filled with gas-free water and situated about 100 cm above the transducer. The probe was then introduced into the longitudinal channel. During this procedure the transducer and pressure probe were flushed continuously with gas-free water from the reservoir. Flushing was discontinued when the probe was in place. The superfusion chamber was then superfused at different flow rates and different gaps. At each particular setting the pressure probe was moved by means of a screw drive in steps of 0.1 mm along the channel and the pressure recorded. To check the effect of the probe and on the pressure or pressure gradient within the channel, some measurements were also made with probe

PRESSURE ON FAST AXOPLASMIC FLOW



2 Arrangement for superfusion of a nerve with constant flow. For further explanation see text.

ing diameter of 1.1 and 1.3 mm. The position of the hole in the probe was indicated by black paint and it was found that the hole was at the center of the gap. The pressure was at its maximum. However, the resolution was not sufficient to decide if this was better than 0.1 mm. For reasons of symmetry it is assumed that the position of the gap is at the peak pressure, even if this is not proved.

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Results

Characteristics of the superfusion chamber

The pressure distribution within the superfusion chamber is shown in Fig. 3. For clarity only two sets of conditions are shown. At a given flow the gap size defines the shape of the curve. The curves were analysed with regard to the following three parameters: the maximum pressure value (in mmHg), the distance in mm between the 50% points on the limbs and the steepest slope of the limbs in mmHg/mm. In Fig. 4 the maximum pressures are plotted against different flows for different gap openings. It is apparent from this graph that the smaller the gap and the greater the flow rates, then the greater is the maximum pressure. Values between 10 and 150 mmHg could easily be achieved. While the slope also depends on the flow rate at

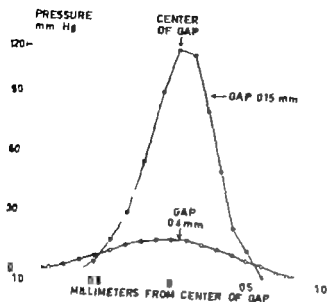


Fig. 3 Pressure distribution along channel of the superfusion chamber. Rate of flow was 390 ml/min, gap 0.15 mm (●) and 0.4 mm (○).

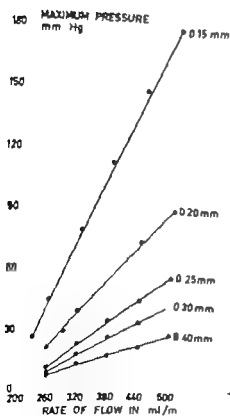


Fig. 4

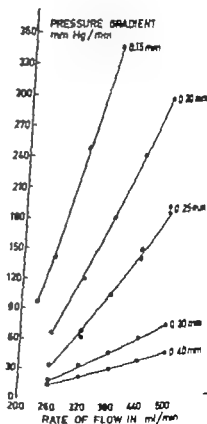
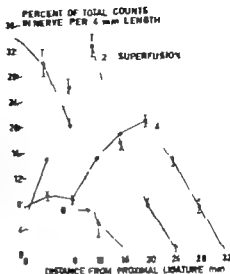


Fig. 5

Fig. 4 Maximum pressure when superfusion chamber is perfused with different rates of flow at different gaps.

Fig. 5 Steepest slopes of the pressure gradients, when the superfusion chamber is perfused with different rates of flow and different gaps.



Distribution of TCA insoluble radioactivity in rat nerve after different times of superfusion at pressure at room temperature. ^{3}H radioactivity was determined immediately after the nerve removed from the animal. 2nd superfusion resp to nerve is superfused for the 1st resp four. Each point represents the mean of 5 expts =

ven gap (Fig. 5) the width is independent of the flow rate and defined by the gap size. The diameter of the pressure probe did not change the results.

The present paper reports results obtained using 0.2 mm and occasionally 0.4 mm gaps.

Rate of transport of tritium labelled TAC insoluble material

Number of experiments were made without compression of the nerve. The distribution of radioactivity along the nerve was determined either immediately after its removal (time 0) after 2, 4 and 20 h. In Fig. 6 the positions of incorporated ^3H labelled material at 2 and 4 h after removal of the nerves are shown. The crest of the 4 h superfused nerve is about 8 mm in dist of the two hour crest. Thus, the rate of transport was about 96 mm/ 24 h. When the nerve was kept for 20 h in a superfusion bath at room temperature the radioactivity was added mainly at the distal ligature with only a small fraction at the proximal ligature. (The size is not shown since the results are almost identical to Fig. 7 a.)

Effect of pressures on fast axoplasmic flow

When a nerve section was subjected to 20 mmHg compression, the movement of labelled material was unaffected (Fig. 7 a). The 20 mmHg compression was achieved either by perfusion of the chamber with a flow of 250 ml/min and a gap of 0.2 mm, or in 2 out of 5 cases with a gap of 0.4 mm and flow of 375 ml/min.

When the pressure was increased to 30 mmHg (290 ml/min, gap 0.2 mm) there was a slight but consistent hindrance of flow within the pressure area (Fig. 7 b). However most of the proteins passed the pressure barrier and collected at the distal ligature.

With 60 mmHg (400 ml/min, gap 0.2 mm) considerable fraction of proteins were arrested when the pressure area. Most of the radioactivity however passed the barrier (Fig. 7 c).

When the pressure was raised to 90 mmHg (500 ml/min, gap 0.2 mm) most of the radio-

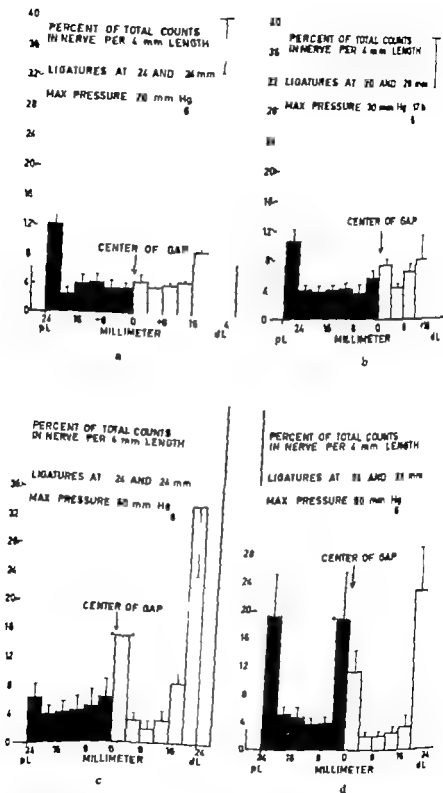


Fig. 7 a, b c, d Distribution of TCA insoluble radioactivity in the vagus nerve 17 h after operation room temperature. Direction of terograde axoplasmic flow. Error indicat $r = 1$ S.E. pL and dL represent the positions of the proximal and distal ligatures resp. Asterisks indicate significant difference between columns (paired t test, $p < 0.05$)

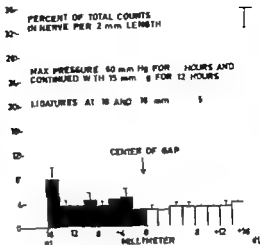


Fig. 8. Reversibility Distribution of TCA-soluble radioactivity in the axon nerve 16 h after ligation at room temperature. Direction of anterograde axoplasmic flow from left to right. PL, proximal ligature; DL, distal ligature. Error indicator = S.E.

ity was blocked at the pressure area, although some passed the barrier and collected at distal ligature (Fig. 7 d).

It is interesting to note that arrested radioactivity in the pressure area was found on both sides of the pressure barrier. At 30 mmHg and 60 mmHg in 8 out of 11 cases the accumulation was more pronounced distal to the pressure barrier while at 90 mmHg this was reversed (5 out of 6 cases).

The accumulation at the proximal ligature was about 10% of the total amount except in 90 mmHg experiments where it reached nearly 20%.

Reversibility of the pressure induced block of FAF

When pressure (60 mmHg) on a nerve section was released 17 hrs after the start, the nerve taken out of the chamber and two new ligatures applied 6 mm proximal and distal to the centre of pressure area. The nerves were then incubated in tissue culture medium TC 199 at 38°C for 5 h using the procedure of McLean *et al.* (1976). In 5 out of 8 cases no excess radioactivity was found in the pressure area. However in three cases there was accumulation indicating that the pressure induced FAF block had not been fully reversed.

When pressure (60 mmHg) was applied for 4 hrs and the nerve then superfused with a flow being less than 15 mmHg, there was no accumulation except at the distal and the proximal ligatures (Fig. 8).

Discussion

In his classical studies of axoplasmic flow Weiss (Weiss and Hallowell 1943) used arterial clamps to compress a nerve; others have utilized spring clips (Denny-Brown and Brenner 1944), tourniquets (Denny-Brown and Brenner 1955; Ochoa *et al.* 1971) or rubber membranes (McLean and Sjostrand 1977). Although some of these studies were not intended to investigate axoplasmic flow the methods have one main feature in common, effects seen may

be due to ischemia or asphyxia rather than to real mechanical obstruction. With the method presented here it is feasible to subject a nerve to adjustable, stable pressures. The concept of constricting a nerve by a flowing solution was first introduced by Birley (1974) and the procedure used here is a further development of his method.

The fact that the pressure within the chamber cannot be recorded continuously during an experiment without interfering seriously with the hydrodynamics, is a disadvantage. However, there was a high correlation between flow and pressure when measured with a pressure probe. This was tested repeatedly for up to 24 h, which was longer than the experimental time. There is no obvious reason why the flow/pressure relation should change considerably when the nerve was substituted for the probe.

It is not known how much of the pressure applied to the surface is transmitted into the center of the nerve. No attempt was made to record the actual pressures within the nerve. If there was an appreciable pressure gradient from the outer to the inner layers due to rigidity of the tissue, the effective pressure would be overestimated (or the degree of block underestimated).

The calculated rate of transport of FAF at room temperature (approximately 22°C) is about a third of the velocity reported by McLean *et al.* (1976) in vagus nerves incubated at 38°C. Axoplasmic flow is temperature sensitive with a Q_{10} of 2-2.3 (Ochs 1971). The rate of flow are thus in fairly good agreement.

The distribution profiles after 70 h incubation at 22°C in the present experiments and incubation at 38°C (data from McLean *et al.* 1976) are different. At room temperature there was about twice as much accumulated at the distal ligature than in the nerves incubated at the higher temperature. At the proximal ligature this relation was reversed. This is most likely due to the temperature dependence of retrograde flow. At both temperatures, the main part of the label would reach the distal ligature. But only a small fraction of the retrogradely transported proteins reached the proximal ligature at room temperature, i.e. only those which were reversed en route while at 38°C there was enough time for a considerable redistribution before the distal ligature.

The present investigation has shown that axoplasmic flow is highly sensitive to a pressure step. As expected, FAF inhibition is due to a pressure difference, not to the pressure per se. Ochs (1974) found no inhibition of FAF when nerves were subjected to up to 5 atm of compressed air. It is striking that already at a pressure of 30 mmHg some of the FAF was blocked. No block was discernible at 20 mmHg but since there is a noise level in the measurement even this low pressure could conceivably have had an effect. The block increased with increasing pressure. It cannot be decided if the effect observed in the whole nerve reflects an effect on the single axon. It seems quite possible that an all or none effect exists. That some axons are more resistant than others is supported by findings of Quigley and Anderson (1976) in FAF block in the optic nerve of owl monkeys.

The effect of pressure steps was reversible at least up to 4 h. It is not yet understood why some nerves did not show reversibility after 15 h pressure. This problem is of obvious importance in connection with reversibility of field changes in glaucoma.

Radioactivity accumulated on both sides of the pressure barrier though it was differently distributed at different pressures. At 30 and 60 but not at 90 mmHg the heaviest accumu-

was found immediately distal to the pressure barrier both when the results were pooled, and in measurements from the single nerve, although not in all cases. Could this be due to a methodological error? It is assumed that the maximal pressure on the nerve is exerted at the centre of the gap, although this could not be proved. Could the maximal pressure may have been at centre, due to slight differences in the shape of the screws. However the pair of screws was randomly selected out of six identical screws for each new superfusion and the direction of the chamber was turned right or left by chance. A methodological error seems unlikely and the observation must thus be regarded as a characteristic feature of axoplasmic flow.

When the pressure was 90 mmHg, axoplasmic flow was strongly inhibited immediately proximal to the pressure barrier and only relatively little protein was able to pass the pressure barrier and collect distally to the pressure barrier. It is interesting to note that the accumulation of protein proximal to the pressure barrier was somewhat higher in the 90 mmHg experiment than in those at 30 and 60 mmHg. It is a well known feature of FAF that components reaching a ligature are reflected and move in retrograde direction (Fruzzell 1974). The finding at 90 mmHg pressure suggests that some protein was reflected at the pressure barrier and moved retrogradely towards the proximal ligature.

The finding that the accumulation of protein is more pronounced distal to pressure barrier at 60 mmHg may be due to accumulation of retrogradely migrating proteins, which had reached the pressure barrier in the anterograde direction and then became reflected en route and now accumulated distally to the pressure barrier. However the phenomenon is not yet fully understood.

In chronic simple glaucoma 30 mmHg intraocular pressure can be deleterious to the optic nerve. The present study shows that pressure in this range is already able to affect fast axoplasmic flow albeit in peripheral nerve and at room temperature.

The superfusion chamber set-up including the electronics was constructed by Folke Hogberg and Claes-Jesper Eriksson. The author gratefully acknowledges The author also thanks Maria Carlsson for excellent technical assistance. The investigation was supported by grant EYE 00231/83 from the National Eye Institute, U.S.A. to Professor E. Békésy.

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Vasopressin release induced by hemorrhage in the goat

By

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Abstract

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of moderate hemorrhage were studied in normovolemic and hydrated conscious goats. Plasma vasopressin (AVP) in jugular vein blood did not rise in response to bleeding of 8 and 11 ml/kg. After blood loss of 18 ml/kg an abrupt and conspicuous rise in plasma AVP (to >10 times basic) was seen in 50% of the experiments. In cases no rise in AVP occurred in the remaining experiments. Bleeding of 20 and 24 ml/kg gave more consistent AVP response. Hydration did apparently not reduce AVP-response to bleeding. The plasma vasopressin concentration returned to pre-hemorrhage about 3 h after bleeding, 1 h before the blood was re-transfused. A rapid and inconsistent rise in plasma activity occurred in response to bleeding. Central venous pressure fell during hemorrhage and stayed depressed until the blood was re-transfused. Since the depression was of the same order in all cases, and was still present when plasma AVP had returned to pre-hemorrhage level, the fall in pressure was obviously not the main cause of the AVP-response. However, the pronounced rise in plasma vasopressin was correlated to fall in the arterial pressure and to hyperventilation. No urge to drink developed during any of the hemorrhage experiments. It is concluded that the goat does not readily respond to hemorrhage with vasopressin liberation. When it happens,pressor accounts of the hormones released, and fall in arterial pressure and/or increased stimulation of respiratory chemoreceptors appear(s) to be the ultimate cause(s) of the release. This thirst-independent regulation of vasopressin secretion is obviously of no importance in day to day control of water balance but may help to maintain the arterial blood pressure in emergency situations.

Words: hemorrhage, vasopressin, thirst, plasma renin activity, arterial blood pressure, central venous pressure, goat.

A release of vasopressin (antidiuretic hormone) from the neurohypophysis is influenced well by the composition as by the volume of the extracellular fluid (ECF). Secretion of hormone in response to normal, or elevated ECF [Na⁺] (and osmolality) is apparently turned by sodium sensitive receptors which predominantly are located near the anterior horn of the third cerebral ventricle (cf. Anderson 1977). The volume-dependent regulation, on the other hand, by all probability consists of both reflex and humoral links. Impulses from cardiovascular distention receptors and baroreceptors appear to exert a tonic inhibitory

influence upon the neurohypophyseal liberation of the hormone (cf. Gauer *et al.* Behn 1970), whereas hypovolemia-induced renal renin release, with subsequent liberation of angiotensin II may stimulate vasopressin secretion (cf. Severs and Daniels-Sem 1971). Such an effect of angiotensin II appears to be mediated by the same cerebral receptors that are affected by the extracellular $[Na^+]$ (cf. Andersson 1977).

In support of the idea that juxtaventricular sodium sensitive receptors play an important role in the regulation of vasopressin secretion is the observation that reduction of the cerebrospinal fluid (CSF) $[Na^+]$ (induced by intracerebroventricular administration of sodium-free solutions) elicits a water diuresis in non-hydrated normovolemic goats (Eriksson 1977). Accordingly prolonged (3 h) infusions of glycerol solution into the lateral ventricle of goats were recently found to cause a sustained depression of the plasma vasopressin level and a persistent water diuresis (Olsson *et al.* 1978). Since the water diuresis was not compensated for by drinking, it gradually induced pronounced hypernatremia and hypovolemia. However, towards the end of the 3 h glycerol infusions the plasma vasopressin started to rise again in spite of the fact that CSF $[Na^+]$ remained subnormal. Since this "escape" may have been a manifestation of volume-dependent regulation of vasopressin secretion, it came of interest to study to what extent hemorrhage stimulates vasopressin release, its liberation, and thirst in the goat.

Methods

Animals. 8 horned female goats (b.wt. 35–55 kg) were used in a total of 26 experiments. The animals were kept in metabolism cages where they had free access to water and were fed hay 3 times a day. In the afternoon the goats received 300 g of commercial grain mix with 6 g of NaCl added.

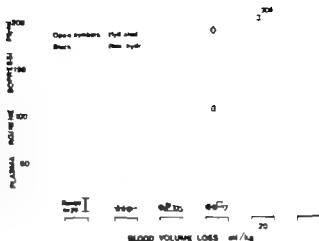
Surgery. In 4 of the goats a permanent polyvinyl catheter was inserted into the carotid artery and a superficial temporal artery under general anesthesia (mebumal). To avoid clotting, the catheters were flushed once daily with isotonic saline and were then filled with heparin solution (1 000 IE/ml).

Experimental procedure. In the morning before an experiment the animals were allowed to feed in the dark until about 10 o'clock. It was then placed in a modified Pavlov stand which prevented it from lying down. Downwards head movements were largely prevented by connecting (near a leather collar) the horns to a bar above the head. However, the goat could still move its head rather freely from side to side. The animal was not allowed to eat when placed in the Pavlov stand but was offered water even in the morning. A cannula (to be used for bleeding and taking of blood samples) was inserted into one jugular vein. In 12 experiments a polyethylene catheter for recording of central venous pressure was inserted into the contralateral jugular vein to a level about 5 cm in front of the right atrium. After control blood samples had been taken for analyses, the goats were bled 8, 12, 16, 20 and 24 ml/kg at a rate of approximately 1 ml/kg/min. The blood was collected in heparinized glass bottles and was re-hydrated to the animals towards the end of the experimental period. In 14 of the experiments the goats were hydrated. They then been given 100 ml/kg of 38°C water by stomach tube into the rumen about 90 min before bleeding started.

Recording of blood pressure and respiratory rate. The carotid blood pressure was measured in the animals via the permanently implanted catheter which was then connected to a Statham transducer. The systolic/diastolic pressures were recorded on a polygraph. Central venous pressure was recorded in the same manner in the jugular vein catheter. When not registered during recordings of carotid blood pressure the heart rate was determined at intervals by auscultation. The respiratory rate was simply determined by counting the expirations.

Blood and urine sampling. Blood for hormone radioimmunoassays was collected in the jugular catheter and was drained into ice-chilled centrifuge tubes with 0.3 M Na EDTA as anticoagulant. The blood was immediately centrifuged at +4°C whereupon the plasma was stored at -20°C until radioimmunoassays were performed. Samples for determinations of hematocrit, (Ht) and Plasma $[Na^+]$, $[K^+]$ and creatinine were collected in heparinized tubes, whereas blood for serum protein determination was taken in glass

Effect of graded hemorrhage on plasma arginine vasopressin (AVP) 45 min after onset of bleeding in 8 hydrated or non-hydrated goats. Each animal is identified by its own symbol, values of bleeding are 8 to 24 ml/kg (approximately rate 1 ml/min). Note the abrupt rise in AVP which occurred as the plasma volume decreased and this at one animal (goat) did lead even to blood loss at 1 kg.



Without anticoagulants. Urine was collected in 30 min samples in Foley catheter inserted into the urinary bladder.

Analyses. Determinations for plasma arginine vasopressin (AVP) were made according to a method previously described by Fyhrquist *et al.* (1976 b). The sensitivity of this method is 0.2 pg/ml. Radioassays of plasma renin activity (PRA) were made according to Fyhrquist *et al.* (1976 a). Plasma $[Na^+]$ and $[K^+]$ are measured by external standard flame photometry using an IL 343 flame meter AA Advanced Instruments Inc. osmometer as used for determinations of the osmolality of fluids. The H values were obtained by centrifugating (in triplicate) microhemocrit tubes at 2000 rpm for 5 min. Serum protein was determined by the Biuret method. Values including \pm figures represent means and S.E. Student's *t*-test as employed for the statistical analyses.

Results

Vasopressin release and plasma renin activity

AVP concentrations observed in jugular vein plasma 45 min after the start of the 8 to 24 ml/kg bleeding periods are shown in Fig. 1. Five of the goats were bled 8 and/or 12 ml/kg (n = 10). On no occasion did this moderate blood loss induce any noticeable elevation of the plasma vasopressin level. In subsequent experiments the same 5 animals were bled 16 and/or 24 ml/kg (n = 11). The effect on plasma AVP was either conspicuous (range 30 to 102 pg/ml, n = 5) or negligible (range 2 to 9 pg/ml, n = 6). In one of the goats even the blood loss of 24 ml/kg was ineffective in raising the AVP. Another animal was bled also 24 ml/kg. The AVP in plasma AVP was then not much greater than that obtained at 20 ml/kg and n = 1 of the 16 ml/kg expts (50 as compared to 30 and 47 pg/ml). Three goats, bled also at 16 ml/kg (n = 3), all responded with large rises in the plasma vasopressin concentration (60 to 191 pg/ml). When greater blood loss was induced in one of these animals (20 ml/kg) the AVP rose to 303 pg/ml. The results presented in Fig. 1 give a general impression that hemorrhage did not cause any graded vasopressin response in jugular vein plasma, but rather an abrupt release of considerable amounts of the hormone when a certain threshold of blood loss had been reached. It is also evident that there were marked interindividual differences in the sensitivity to bleeding, and that the amount of

TABLE I Hemorrhage induced effects on jugular plasma arginine vasopressin (AVP) ($\mu\text{g ml}^{-1}$; Y_{50} (about $1 \text{ ml kg}^{-1} \text{ min}^{-1}$) was started at zero time and lasted for 8 to 26 min. N-R-experiments in which no noticeable rise in AVP occurred. R = AVP-positive experiments. Duration of transfusion 20–35 min.

Time	Hemorrhage				
	8 and 12 ml/kg N-R (n=10)	16 ml/kg N-R (n=5)	R (n=3)	20 and 24 ml/kg N-R (n=1)	R (n=5)
-45	2.6 ± 0.4	4.1 ± 0.5	6.1 ± 1.1	5.9	4.3 ± 2.8
15	2.5 ± 0.4	4.1 ± 0.5	6.4 ± 2.2	4.6	4.1 ± 1.2
	Bleeding				
45	2.8 ± 0.3	3.6 ± 0.6	97.4 ± 25.4	8.9	115.8 ± 46.9
75	2.3 ± 0.4	2.7 ± 0.8	46.1 ± 19.1	8.9	61.8 ± 12.0
105	2.0 ± 0.3	3.3 ± 0.5	30.7 ± 15.9	5.8	39.0 ± 11.1
135	—	—	16.6 ± 6.7	—	14.9 ± 5.9
165	—	—	14.0 ± 5.8	—	$4.9 (n=3)$
195	—	—	7.0 ± 1.9	—	$2.4 (n=3)$
225	—	—	7.6 ± 2.1	—	$2.5 (n=3)$
	Re-transfusion				
5 min after retransf.	2.2 ± 0.6	3.9 ± 0.5	7.7 ± 2.7	5.0	8.2 ± 2.5

vasopressin released was not clearly related to the volume of blood loss. Further hydration was not seen to diminish the hormone release in response to hemorrhage.

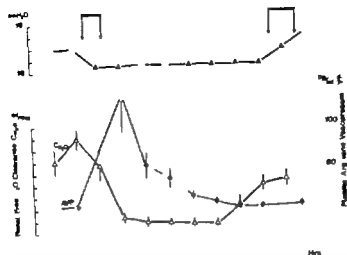
The plasma AVP was determined at 30 min intervals after the first post-hemorrhagic sample had been taken (45 min after onset of bleeding). The results are presented in Table I. Here the "AVP positive" experiments have been separated from those in which no measurable rise in AVP occurred. In the positive experiments the peak level of plasma AVP was always found in the first post-hemorrhagic sample. Plasma AVP then gradually returned to pre-bleeding values. This occurred within 3 h after bleeding, *i.e.* before the last 1/2 volume was transfused back into the animals.

As expected, "AVP-positive" hemorrhage always induced a pronounced and sustained inhibition of the water diuresis of hydrated goats. As shown in Fig. 2 this inhibition of water diuresis was well correlated in time with the rise in AVP. Thus, after having become negative for about 2 h, the renal free water clearance returned to positive values before retransfusion of the blood. In contrast, the course of the water diuresis never became affected when hemorrhage in hydrated goats did not cause measurable increase in plasma AVP.

In most experiments the bleeding caused some increase in PRA (Table II).

Absence of thirst

In none of the 26 hemorrhage expts. did the goats drink the water offered to them at regular intervals. That the animals were restrained to a certain extent does not seem to be the reason for the absence of drinking motivation. Thus, on other occasions two of the goats were deprived of water for 24 h. They were then placed in the Pavlov stand where they were restrained in the usual manner. When offered water both goats immediately drank about 2 l of water.



2. Effects of bleeding (B) (blood loss 16 or 20 ml/kg b wt.) on central venous pressure (CVP), plasma vasopressin (AVP) and renal free water clearance (C_{H_2O}) in 6 AVP-positive experiments performed in 4 hydrated goats (compare Fig. 1). Note that the CVP remains depressed until the lost blood was re-transfused (R), whereas plasma AVP returns to pre-hemorrhage level, and the C_{H_2O} to the value, 40 min earlier. The rate of blood loss was about 1 ml/kg¹ min¹. Vertical bars S.E.

Cardiovascular and respiratory effects

Carotid blood pressure was followed during 111 expts. (4 animals). Hemorrhage causing blood loss of 8 and 12 ml/kg (4 recordings, 3 goats) did not induce any fall in the mean arterial pressure. However, a slight decrease in pulse pressure and about 50% increase in heart rate occurred towards the end of the bleeding periods. Neither did bleeding at 16 and 20 ml/kg cause any fall in the arterial pressure of the goats which did not respond with vasopressin release. In two goats responding with pronounced elevation of plasma AVP to hemorrhage of 16, 20 or 24 ml/kg, a 15 to 25 mmHg fall in mean carotid pressure was seen at the end of bleeding. The drop in mean pressure was accompanied by a marked decrease in pulse pressure and a rise in respiratory frequency from about 15 to about 200/min. The arterial blood pressure and the respiratory frequency did not return to pre-hemorrhage values until about 2 h later. Polyposis of the same intensity and duration also developed in other experiments in which rise in plasma AVP was seen. However no increase in respiratory frequency occurred in the AVP-negative expts.

The central venous pressure (CVP) was followed during 12 expts. (bleeding at 8 and 12 ml/kg, 4 at 16 ml/kg, n = 7 and at 20 ml/kg, n = 1). Bleeding invariably induced depression of CVP which was of the same order in all expts. (about 6 cm H₂O) regardless of whether a AVP-response was obtained or not. The CVP remained depressed until the lost blood plasma was transfused back into the goats. Then the CVP rose above pre-bleeding level (Fig. 2).

Blood composition

There were no noticeable changes in either plasma [Na⁺], [K⁺] or osmolality during the course of any of the expts. Serum protein had fallen slightly (about 5%) 105 min after the

TABLE II. Hemorrhage-induced effects on jugular plasma renin activity (PRA) ($\text{ng ml}^{-1} \text{ h}^{-1} \text{ body}$ (about $1 \text{ ml kg}^{-1} \text{ min}^{-1}$) was started at zero time and lasted for 8 to 26 min. N-R: expts. in which no noticeable rise in AVP occurred. R = AVP-positive experiments. Duration of transfusion 20–35 min.

Time	Hemorrhage			20 and 24 ml/kg	
	8 and 12 ml/kg N-R (n=10)	16 ml/kg N-R (n=5)	R (n=5)	N-R (n=1)	R (n=5)
-45	0.26 ± 0.05	0.21 ± 0.15	0.75 ± 0.31	0.26	0.74 ± 0.25
-15	0.26 ± 0.07	0.34 ± 0.12	0.65 ± 0.30	0.32	0.66 ± 0.15
	Bleeding				
45	0.48 ± 0.09	0.39 ± 0.18	0.81 ± 0.40	0.32	0.69 ± 0.23
75	0.57 ± 0.13	0.39 ± 0.16	0.88 ± 0.37	0.66	0.53 ± 0.17
105	0.51 ± 0.18	0.60 ± 0.39	1.29 ± 0.51	0.24	0.86 ± 0.27
135	—	—	1.69 ± 0.87	—	0.67 ± 0.17
165	—	—	2.11 ± 1.20	—	1.17 (n=2)
195	—	—	1.95 ± 0.95	—	1.39 (n=3)
225	—	—	1.02 ± 0.78	—	1.26 (n=2)
	Re transfusion				
5 min after retransf.	0.36 ± 0.12	0.39 ± 0.25	1.25 ± 0.65	0.18	0.63 ± 0.17

more moderate bleeding (8 and 12 ml/kg). This fall was not statistically significant. However a significant ($p < 0.01$) drop in serum protein was recorded 105 min after start bleeding when the goats were bled 16 and 20 ml/kg (from 6.7 ± 0.1 to $5.8 \pm 0.2 \text{ g/100 ml}$). The reduction in serum protein was of the same order in "AVP-positive" and "AVP-negative" expts. However a difference in the effects on Ht was observed between these two groups of expts. A 14% reduction in Ht ($p < 0.05$) had occurred 105 min after the onset of bleed (16 and 20 ml/kg) in the "AVP-positive" expts, whereas the fall in Ht was only 5% when bleeding at these degrees did not induce a rise in AVP. The 8 and 12 ml/kg bleed caused even less reduction in Ht (about 3%).

Renal salt excretion

No apparent change in renal potassium excretion occurred during any of the expts. However a decrease in renal sodium excretion was observed in response to the blood loss at 16, 20 and 24 ml/kg in the "AVP-positive" expts. Here the Na^+ excretion gradually dropped from $54 \pm 13 \text{ } \mu\text{mol/min}$ to $15 \pm 6 \text{ } \mu\text{mol/min}$ in the third post-bleeding period. It remained at this low level until the blood was retransfused. The corresponding values in the "AVP-negative" expts. were $54 \pm 18 \text{ } \mu\text{mol/min}$ (before bleeding) and $59 \pm 28 \text{ } \mu\text{mol/min}$.

Discussion

Already 40 years ago Rydén and Verney (1938) showed that hemorrhage, accompanied by a transient fall in arterial blood pressure, causes an inhibition of the water diuresis in fasted, conscious dogs. Since the inhibition was of much longer duration than the depression of the blood pressure, they concluded that it was caused by some humoral factor, probably antidiuretic hormone, and not by changes in renal hemodynamics. More recent studies have con-

that antidiuresis, developing in response to bleeding, in fact is due to release of pressin (cf. Share 1969), but the mechanism behind hemorrhage-induced release of the hormone remains a matter of controversy. Experiments in animals and man have provided extensive evidence that cardiovascular distension receptors in the low pressure system are involved in the regulation of vasopressin secretion (cf. Gauer *et al.* 1970). However the importance of such a mechanism in the control of water balance has also been called in question (Goetz, Bond and Bloxham 1975). Studies in rats (Dunn *et al.* 1973), monkeys (Scheidt *et al.* 1977) and man (Goetz, Bond and Smith 1974), performed since radioimmunoassays of plasma AVP became possible, imply that substantial release of vasopressin in response to hemorrhage does not occur until blood has been lost to such an extent that the arterial blood pressure begins to fall. The results of the present study suggest that this also holds true in the goat. Furthermore, the experiments also imply that the observed AVP response to bleeding was not due to reduced inhibitory inflow from distension receptors in the low pressure side of the cardiovascular system. In the experiments where the arterial pressure was recorded, a rise in plasma AVP was only observed when bleeding temporarily caused hypotension, and the blood pressure never fell when recorded in "AVP-negative" hemorrhage experiments. The CVP, on the other hand, was reduced to the same extent as in "AVP-negative" as in AVP-positive expts., and remained depressed even when plasma AVP had returned to pre-hemorrhage concentrations (Fig. 2). It could be argued that the CVP was recorded in the anterior caval vein and not in the left atrium, which is considered the principal location of distension receptors involved in the regulation of vasopressin secretion. However it has been demonstrated (Henry Gauer and Sjeker 1956) that, within changes of 30% of the blood volume, the pressure in the large thoracic veins rises and falls in parallel with the atrial pressures. That the observed conspicuous elevation of plasma AVP should have been due to reduced activity of distension receptors in the low pressure system is unlikely also for another reason. The rise in plasma AVP had more the character of an all or none response (Fig. 1) with no clear-cut relation to the volume of blood lost. A much more graded AVP response would have been expected if the vasopressin release had been a manifestation of reduced blood volume in the low pressure system. It is likely that blood obtained from the jugular vein shows higher absolute vasopressin concentration than that seen in peripheral blood. Thus, even minor changes in AVP-concentration ought to have been detected in this study. Even though a fall in arterial pressure apparently had to occur before hemorrhage induced substantial vasopressin release, this must not necessarily mean that reduced baroreceptor inflow was the crucial factor. In the present experiments the AVP response invariably was accompanied by pronounced hyperventilation. No measurements of arterial pO_2 , pCO_2 or pH were made, but it appears most likely that the hyperventilation was mainly a manifestation of hypoxia. Forsling and Ulfman (1976) have shown in anesthetized dogs that hypoxia increases vasopressin secretion, presumably by an effect on receptors at the medullary level. Therefore, it appears likely that diminished O_2 -transporting capacity of the blood during bleeding might have contributed substantially to the large rises in plasma AVP seen in many of the present expts. Of interest in regard to the possibility that hypoxia may have influenced or contributed to the hemorrhage-induced elevation of plasma AVP is also the

differential effects on hematocrit. In the "AVP-positive" expts. bleeding caused a considerably greater fall in hematocrit than did the corresponding blood loss in AVP-negative expts. Possibly the AVP-sensitivity to hemorrhage was negatively correlated to the amount of stored erythrocytes available for compensation of the blood loss. However, the sharp fall of Ht in the "AVP positive" expts. may also have been a manifestation of homeostatically induced water retention.

The hypovolemia induced in the present animals caused only a minor rise in PRA. It appears most unlikely that this weak and inconsistent activation of the renin-angiotensin system (Table II) could have contributed significantly to the conspicuous vasopressin release seen in the "AVP-positive" expts. Thus, the vasopressin release which is obtained in response to infusions of rather unphysiological amounts of angiotensin II into the third cerebral ventricle of the goat is apparently of much smaller order than that observed in the present study (Lishajko and Andersson 1975). Furthermore, since angiotensin is a powerful dipsogen (cf. Severs and Summy Long 1975) thirst ought to have developed in the "AVP positive" expts. if activation of the renin-angiotensin system had been the ultimate cause of the pronounced vasopressin effect. The fact that no urge to drink developed in response to bleeding (sometimes followed by an almost 100-fold elevation of jugular plasma AVP, Fig. 1) shows that an emergency regulation of vasopressin secretion exists, which is largely independent of the regulation of water intake. It is in agreement with previous studies in other species which have shown that thirst is by far no consistent effect of rather substantial blood loss (cf. Fitzsimons 1972). The purpose of this, apparently thirst-independent, release of huge amounts of AVP could be to assist in the maintenance of the arterial blood pressure in emergency situations. As previously pointed out (Goetz *et al.* 1974) that mechanism is obviously of no importance in day to day control of water balance. The homeostatic control of water balance demands a much more subtle regulation of vasopressin secretion which is intimately linked to the regulation of water intake. This has recently been illustrated in the goat by measurements of plasma AVP during developing dehydration (Olsson *et al.* 1978). During two days of water deprivation the plasma AVP gradually rose concomitant with elevation of plasma [Na⁺]. However, the AVP level in jugular vein plasma at the end of the dehydration period was only about 30% of that observed here in the "AVP-positive" expts. (Table I). This in spite of the fact that the two days of water deprivation had caused a net loss of body fluid which was several times that induced by the present hemorrhage.

Taken together the results presented here and the earlier vasopressin-data obtained in dehydration experiments (Olsson *et al.* 1978) would indicate a dual role for vasopressin: one of importance in water economy and the other with the purpose of maintaining blood pressure in sudden hypovolemic hypotension.

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Simultaneous measurements of capillary diffusion and filtration exchange during shifts in filtration-absorption and at graded alterations in the capillary permeability surface area product (PS)

By

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Abstract

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The diffusion exchange of Cr-EDTA, using the single injection indicator diffusion method, was determined simultaneously with estimations of the capillary filtration capacity (CFC) in an 'isogravimetric' set-up during quarter preparation during artificial perfusion and maximal dilatation. Measurements were performed at constant flow and during 1) shifts in filtration-absorption, 2) alterations of perfused capillary area (graded rarification of capillary network by microsphere injection) and 3) during alterations of permeability (i.e. infusion of histamine). At maximal vasodilatation CFC was 0.037 ± 0.001 ml/min \cdot mm Hg \cdot 100 g. PS for Cr-EDTA 5.67 ± 0.13 ml/min \cdot 100 g. During filtration or absorption, Cr-EDTA transfer to vessels to interstitium changed only slightly but the situation may well be different for solute transfer to interstitium to vessels. Alterations in capillary wall area resulted in proportional changes in PS for Cr-EDTA while the CFC changes were always relatively smaller. Histamine increased CFC some threefold with a marked increase in protein transfer while PS for Cr-EDTA increased only marginally. This inotropic effect could be ascribed mainly to an increase in the number of large pores which, because of its relative paucity, are of little importance for small molecular diffusion exchange but highly important for convective and macromolecular exchange.

Concerning capillary diffusion and filtration exchange, only few attempts have been made to measure simultaneously and quantitatively both these processes under well controlled conditions and with independent techniques. Since transcapillary exchange of water-soluble substances is considered to take place not only by pore-bound diffusion but also to a variable extent by convection, the sparsity of such studies may seem surprising which, however, to a great extent is due to the considerable technical difficulties involved. In general it is accepted that the rapid transcapillary passage of lipophilic and small hydrophilic substances occurs mainly by diffusion, while the slower net shift of fluid between the intra- and extravascular spaces takes place by convection (cf. Landis and Pappenheimer 1963). However, convection may well be of dominant importance also for the blood to tissue transport of macromolecules.

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like plasma proteins, because recent data suggest that both diffusion and micro-circulation may contribute very little to this particular transfer (e.g. Lassen, Parving and Jørg 1974, Rippe, Kamiya and Folkow 1977, Lassen 1978, Rutli 1978). Earlier studies on the interrelationships between diffusion and convection as mechanisms under for small hydrophilic substances through the capillary membrane are, however, not in part conflicting. Data by Kruhof (1946), Pappenheimer, Renkin and Borrero (1951), and more recently by Fleming and Diana (1976) suggest that the effects of convection on blood to tissue transfer of most such solutes is negligible, but this view has been questioned by e.g. Åberg (1973). Further transport in the opposite direction, relevant to the elimination of tissue-produced metabolites, appears at least in skeletal muscle to be considerably augmented by convection, ascribed mainly to a pericapillary 'micro-circulation' influence (Lundgren and Mellander 1967). It should here be realized that most studies of blood to tissue transport usually deal with the transcapillary passage only while experiments concerning the opposite route of transfer includes the interstitial space as well. Particularly in skeletal muscle this implies passage along narrow intercellular clefts over considerable distances, since capillaries are here 40-50 μm apart, even when all vessels are open to flow which only occurs during intense exercise and at maximal vasodilatation. With respect to shifts in perfused capillary surface area in connection with vasomotor transients, changes in capillary filtration capacity (CFC) and diffusion capacity (PS) have mainly been followed in separate investigations (e.g. Renkin and Rowell 1962 a, b, Boldt *et al.* 1963, Kjellmer 1964, Renkin, Hudlicka and Sheehan 1966). Only in few studies the two parameters have been simultaneously measured with entirely independent techniques, as in the small intestine (Dresel, Folkow and Wallentin 1966). In the classic capillary studies by Pappenheimer and co-workers (*cf.* Landis and Pappenheimer 1963) diffusion and filtration capacities were followed in close parallel by means of the isogravimetric technique modified to allow concomitant measurements of osmotic transients, later adopted also by Diana, Long and Yao (1972) and Diana and Laughlin (1974). This ingenious osmotic transient method is, however, now known to have certain limitations inherent in the fact that osmotic transfer of water occurs also across the endothelial cell membranes, which was here balanced off by a filtration that is restricted largely to the interendothelial clefts. Fortunately this error was at least partly offset by the fact that no account was taken of the reflection coefficient of the test solutes, which happens to introduce an error acting in the opposite direction (*cf.* Crone and Lassen 1970, Perl 1971). Concerning alterations of capillary permeability only few simultaneous measurements of CFC and PS have been performed, where Diana, Long and Yao (1972) used histamine but observed only small histamine effects on either PS or CFC. More recently Rippe and Grega (1978), using the presently described *in vitro* perfusion technique, observed marked increases in CFC after histamine but only minor increases in PS, results that will be further dealt with below.

The present study was performed in order to follow simultaneously and quantitatively the blood to tissue transfer of Cr-EDTA (Mw 341) and the capillary filtration capacity (CFC), using a colorimetric on-line variant of the single injection double indicator diffusion method (Rippe and Stage 1976, 1978) together with a variant of the isogravimetric

method (Pappenheimer and Soto Rivera 1948). The study was performed on the maximally vasodilated, artificially perfused rat hindquarter preparation in which pressures, flow and other hemodynamic parameters could be precisely controlled (Folkow *et al.* 1974). Setting out from such well controlled experimental conditions a series of graded changes in flow, pressure and capillary characteristics were induced, exploring the quantitative and simultaneous effects on PS and CFC. First PS for Cr-EDTA was followed over a wide range of flows during maximal vasodilatation when capillary surface area and permeability were kept largely constant because the frequently observed increase in "apparent PS" with increasing flow is still a controversial phenomenon. Measurements were then made at constant flow but during three principally different and selective changes in capillary function: 1) shifts in filtration-absorption; 2) alterations of perfused capillary wall area caused by graded rarifications of the capillary network, and 3) alterations of capillary permeability caused by histamine infusion. In this way models of the physiological more important variables in capillary exchange function could be separately explored as to their effects on diffusion and filtration events, under circumstances where most other parameters could be held largely constant. Part of the results have been briefly reported earlier (Rippe, Kamiya and Folkow 1978).

Methods

Preparation and technical arrangements. Experiments were performed on the isolated hindquarters of male Wistar albino rats, weighing between 270 and 380 g. The hindquarters were arranged in the "isogravimetric method" (cf. Pappenheimer and Soto Rivera 1948; Ellassen *et al.* 1974) and for a semimetric on-line modification of the single injection indicator diffusion method (Rippe and Stage 1971, 1978).

The isogravimetric rat hindquarter preparation has been described elsewhere (Folkow *et al.* 1974). Briefly the tail artery was cannulated during ether anesthesia for continuous mean arterial pressure recordings, after which the animal was further anesthetized with Nembutal[®] 3 mg/100 g, and chloralose. The abdominal aorta and the inferior caval vein were freed from the level of the renal vessels to the lumbar ones. Then the hindquarters were isolated by mass ligatures just proximal to these latter vessels and the spinal channel and bone marrow were plugged with cotton wool soaked in silicone. Tail and penis were excluded by tight ligatures so that the preparation consisted mainly of skeletal muscle (70–75%).

After heparinization the abdominal aorta was cannulated and connected to an artificial perfusion system driven by Harvard perfusion pump. The caval vein was connected to a venous outflow cannula while one of the renal veins was used for continuous venous pressure recordings (P_V). The completed isolated hindquarter preparation was then placed on a balance plate connected to a strain gauge for continuous weight recordings. Finally the venous outflow cannula was coupled in series with a pH electrode and a densitometer system for simultaneous recordings of Cardio-Green coupled to albumin and Cr-EDTA. The arterial inflow cannula was side branched to a step dispenser syringe for injection of the mentioned two indicators used for measurements of capillary diffusion events (cf. Rippe and Stage 1971). The free end of the venous outflow cannula, i.e. the one distal to the densitometer system, could be adjusted to any desired level to set the venous outflow pressure P_V . Flow and P_A could be controlled by adjusting the setting of the perfusion pump. Tissue weight P_A and P_V were continuously monitored on a Grass polygraph, while pH and the dye dilution curves were recorded on a potentiometer writer.

4 per cent dextran (Macrodex[®] Mw about 70 000 supplied by AB Pharmacia Sweden) in Tyrode solution was used as perfusate to which was added horse serum (Normal Serum, SBL, Sweden) 100 ml/l. The perfusate was gassed with a mixture of 97% O_2 and 3% CO_2 and kept at 38°C. Perfusion flow was kept constant at 11–14 ml/min 100 g of tissue throughout the experiment except when the effect of flow on PS was studied. Maximal vasodilatation was maintained during the whole experiment by means of slug injections (9.5 ml at a time) or a slow infusion of papaverine (20 µg/ml perfusate).

Capillary filtration and diffusion were followed by frequently repeated measurements, sometimes even

small molecular permeability was assessed by monitoring PS for Cr-EDTA by the colorimetric modification of the single injection indicator diffusion method (Rippe and Stage 1978). Capillary hydraulic conductivity was determined as capillary filtration coefficient (CFC, cf. Ekmann *et al.* 1974). Changes in large molecular permeability influencing the effective colloid osmotic pressure difference across the capillary walls, were reflected by the consequent changes in capillary fluid equilibrium as they occurred *in situ*, even though the hydrostatic capillary pressure was kept constant.

Experimental procedures: Filtration/absorption as induced either by graded alterations of capillary static pressure by means of changing venous pressure, or by graded changes of the crystalloid osmotic pressure by means of infusion of hypertonic saline, transiently increasing the crystalloid osmolarity from about 290 mosm/l to around 330 mosm/l at maximum. Osmotic transients of up to 30–40 mmHg were thereby created, implying reflection coefficient for saline in the order of 0.62–0.65. More systematic experiments in this respect are, however, planned in subsequent study.

To assess the effects on PS of changes in capillary hydrostatic pressure the following procedures were used:

After 2 to 3 control measurements of CFC and PS for Cr-EDTA during complete isogravimetry (no pressure as elevated up to 30 mmHg at maximum, and after about 1 min of steady filtration PS for Cr-EDTA was measured once or twice. Flow was kept constant throughout these manoeuvres which did not raise the arterial pressure. After 3–7 min of filtration, venous pressure was lowered to the control value and now absorption ensued at slightly lower rate than the former filtration. During this absorption phase, PS for Cr-EDTA was again measured and this parameter was now followed until isogravimetry is reestablished. Here additional control measurements are performed.

Implying changes in the capillary crystalloid osmotic pressure the procedures were as follows. After several measurements of CFC and PS for Cr-EDTA during isogravimetry infusion of hypertonic saline (4.5 ml/min of 4 solution) started, upon which the preparation begins to absorb fluid across the capillary walls until new isogravimetric equilibrium is established. When the hypertonic infusion is stopped, filtration ensued until the former isogravimetric fluid equilibrium was reestablished. PS for Cr-EDTA was followed during the whole procedure, during the absorption phase isogravimetric phase the subsequent filtration phase. In some experiments also CFC was measured during the saline infusion period to check that the hyperosmolality did not alter the capillary hydraulic conductivity.

In order to reduce the perfused capillary surface area in the osmotically vasodilated preparation, different sizes of microspheres (ϕ 15 μ m) were given as bolus injections intra-arterially. In every experiment it is thus each injection was given so as to induce graded alterations in the perfused capillary surface area.

Immediately after the injections there occurred fairly abrupt decrease in arterial pressure while venous pressure remained constant, and as consequence of the arterial pressure decrease and the constant venous conditions the hydrostatic pressure rose in the perfused capillaries. Therefore, slow filtration started with short latency (cf. Kuriya, Rippe and Folkow 1978). PS for Cr-EDTA, CFC and PRU₃₀ were measured at every level of plugging. By lowering venous pressure, filtration could be avoided during the measurements.

In order to induce changes in capillary permeability histamine was infused intra-arterially in the osmotically vasodilated preparation in supra-analgesic doses (20–40 μ g/ml perfusate). PS for Cr-EDTA and CFC were measured 2–3 times after about 5 minutes of histamine infusion, which caused steady weight increase.

Calculations: The principles for the computer treatment of the *in situ* recorded tracer curves have been given earlier (Rippe and Stage 1978). Instantaneous extraction values (E) and integrated values (area under the curve) of Crone and Lassen (1970), plotted against time, were obtained for every set of single injection curves. E_{max} integrated up to the time when the tracer curve had declined to 30% of peak values was used for calculations. The PS product was calculated according to the formula $PS = Q_p \ln(1/E)$ (Renkin 1959, Crone 1963) where Q_p is the measured plasma flow. This formula is valid only if capillary plasma inflow and outflow are equal. Such is, of course, not the case during capillary net filtration and absorption.

In three cases simple modification of the Renkin-Crone formula was employed, based on the assumption that the capillary outflow concentration of the non permeant tracer only or alternatively that of both the non-permeant and permeant tracer is changed by the factor Q/Q' during convection, where Q' is the capillary inflow and Q the capillary outflow of perfusate. The following expressions are then obtained:

$$PS = \frac{Q E \ln \frac{Q}{Q'} (1-E)}{1 - \frac{Q}{Q'} (1-E)} = \frac{Q - Q'}{2} \ln(1-E)$$

The expressions reduce to the simple Renkin-Crone formula if Q is set equal to Q_p . The difference between the exact formulation (left) and the approximate one (right) is at maximum 0.5% for the flow rates and extraction values of this study which was evident from a simulation procedure when Q and E were changed over a wide range.

As also the non-permeant tracer is slightly permeable through the capillary, all correction had to be made for this. The filtration-dependent transcapillary plasma clearance of albumin (Cl_{alb} , ml/min/100 g) during dextran-horse serum perfusion at maximal vasodilatation has earlier been determined to

$$Cl_{alb} = 0.03 + 0.43 J_v$$

where J_v is the net filtration rate (Rippe and Folkow 1977). As this relation was known, the exact E of albumin could easily be determined at every filtration rate at known perfusate flow (Q_p) from the value $Cl_{alb} = Q_p E_{alb}$. The measured extraction values for Cr EDTA at peak time E_{peak} (see Fig. 2), were then corrected according to the formula

$$E_{corr} = \frac{E_{alb} + E_{peak}}{1 + E_{alb}}$$

The ratio E_{corr}/E_{peak} was used as a correction factor for E_{peak} . Corrected E_{peak} was then substituted in the modified Renkin-Crone formula above. Though negligible at low filtration rates, this correction becomes increasingly important at high filtration rates.

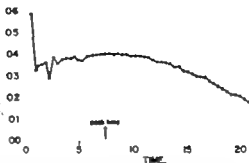
Correction for histamine induced transcapillary leakage of the non-permeant tracer was not made in this study since no experimental data were available for such a correction. However the error of making this correction could be roughly estimated from the data on albumin clearance during histamine infusion by Appelgren, Jacobsson and Kjellmer (1966) and on control albumin clearance at histamine infusion during dextran-horse serum perfusion (see above) and pure horse serum perfusion (cf. Rippe and Folkow 1977, Rippe, Kamiya and Folkow to be published). The results of the present study are also valid according to the following considerations. Albumin clearance by large pore diffusion was assumed to increase from around 0.005-0.01 ml/min/100 g (Rippe, Kamiya and Folkow to be published) to about 0.3 ml/min/100 g by histamine and the weighted reflection coefficient for the whole capillary membrane to decrease from around 0.5 to around 0.15-0.20 (see Results). Dextran-horse serum perfusion alone induces a decrease in the capillary reflection coefficient for albumin from around 0.90-0.95 during perfusion with pure horse serum (Pappenheimer and Soto R vera 1948) to around 0.5 (Areskal 1969, Rippe and Folkow 1977) probably by affecting the small pores without much changing their dimensions (cf. Rippe and Folkow 1977). Based on such considerations albumin clearance during histamine infusion was estimated to $Cl_{alb} = 0.3$ ml/min/100 g where the symbols were defined above. The error of not correcting the E values according to this estimate results in an underestimation only of some 7-15% in the present study. Most PS measurements during histamine infusion were performed at lowest possible filtration rates (around 0.5 ml/min/100 g) which is brought about by e.g. lowering venous pressure.

Results

1. Apparent fractional extraction as a function of time

In most experiments the apparent fractional extraction Cr EDTA versus time showed a rapid initial rise, directly followed by a transient, dip-like decrease, but at the time when the tracer curves reached their peaks ('peak time') a plateau of some seconds duration was obtained, followed by a more gradual decrease (Fig. 1). In some 40% of the experiments the initial apparent fractional extraction value was slightly higher than that at peak time (E_{peak}). In 30-40% it was approximately the same and in around 30% it was somewhat lower. Maximum value of the overall extraction, calculated by the area method (E_{area}) used in this study (cf. Rippe and Stage 1978) was some 5-8% lower than the maximal instantaneous extraction (E_{max}) but roughly proportional to it (Fig. 2). Thus in this study

EXTRACTION



Computer plot of apparent fractional extraction of Cr-EDTA vs. time from individual experiments.

relative changes in area extraction, or in PS calculated from E_{area} paralleled the changes in E_{max} which, on the basis of computer modelling, has been proposed to be the best approximation of true overall extraction (cf Basingthwaite 1974). However E_{area} was in the present study preferred to E_{max} because of its higher reproducibility in the given experimental situation. Thus the coefficient of variation between sequential E_{area} determinations was only between 2 and 3% while that of E_{max} was around 5%.

Apparent PS as function of perfusate flow

Although the vasculature was maximally dilated and thus all capillaries were open to flow, as also judged from CFC determinations, apparent PS varied with perfusate flow in the experimental range as shown in Fig. 3. However at flows above 11–12 ml/min 100 g PS remained relatively constant, even though a slight tendency of increase was seen also here. For such flows the PS determinations, performed after induced changes in capillary function, were performed at flows above 11 ml/min 100 g and flow was in the individual experiment kept constant throughout so that the PS determinations should not be disturbed by flow changes. At a mean flow of 12.5 ± 0.3 ml/min 100 g and a mean E_{area} of 0.365 ± 0.001

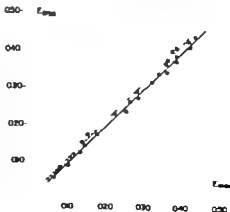


Fig. 2. Relationship between maximal extraction (E_{max}) and area extraction (E_{area}), interpreted up to the time when the tracer curves had declined to around 50% of their peak values in 9 randomly selected expts. The data are taken from control recordings during maximal vasodilatation, as well as from recordings during the different test manoeuvres of this study. The unity line (dashed) is shown together with the line of the linear regression of E_{max} on E_{area} described by the equation $E_{max} = E_{area} + 0.975$.

The expressions reduce to the simple Renkin-Crone formula if Q is set equal to Q' . The difference between the exact formulation (left) and the approximate one (right) is at maximum 0.5% for the flow rates and extraction values of this study which was evident from a simulation procedure when Q and E were changed over a wide range.

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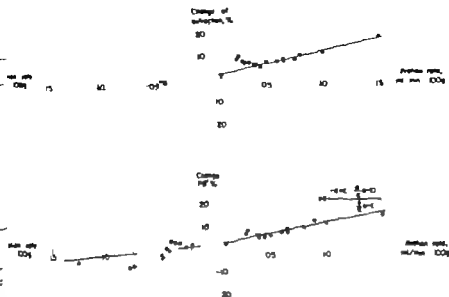
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4 The relative changes in extraction (E) and apparent PS for Cr-EDTA at graded alterations in capillary absorption. Filled circles are from experiments in which convection was induced by changing intercapillary pressure, and open circles from experiments in which such alterations were induced by graded intracapillary osmotic pressure. The dashed line (see upper part) shows the change in extraction ΔE is caused by convection-induced differences in (arterial) inflow to and (venous) outflow from the tissue vessels. The changes in transport-constant for Cr-EDTA are of the order of magnitude as predicted from the theory of irreversible thermodynamics.

where J_s is the measured unidirectional flux of solute, J is the filtration rate, \bar{c} the mean capillary solute concentration and σ the reflection coefficient of the solute. Interstitial concentration is here supposed to be zero. The first term of this expression gives the pure convection flux and the second one the filtration flux. The obtained data are well compatible with this expression for a σ of Cr-EDTA in the order of 0.05–0.15 and for a mean solute concentration in the pore that is higher than $\bar{c}/2$ during filtration and lower than this value during absorption (cf. Grotte 1956). No attempt was made to calculate σ because the statistical errors inherent in the data will then be amplified in an unacceptable manner.

The extraction values during filtration-absorption are affected both by pure convection effects on total transport (see above) and by convection induced concentration/dilution effects, causing artefactual changes of the venous concentrations of permeant and non-permeant tracer. In Fig. 4 this artefactual relative change in E is represented by a dashed line, which was calculated according to the formula

$$\Delta E = \left\{ 1 - \exp \left[\frac{PS}{Q - \frac{J_v}{2}} \right] \right\} / \{ 1 - \exp(-PS/Q) \}$$

where J_v is the volume flux through the capillary membrane and $(Q - J_v/2)$ represents the mean perfusate flow through the system (see Methods). The numerator represents the E value corrected according to the modified Renkin-Crooks formula used in this study (see

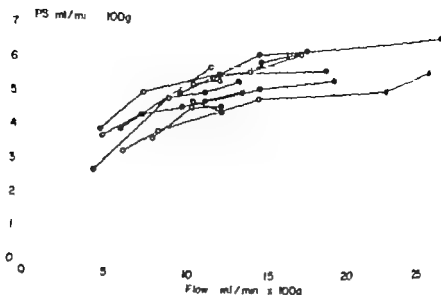


Fig. 3 Apparent PS for Cr EDTA in the maximally vasodilated hindquarter vascular bed placed at flow in 13 μ tu. Values from the same expt. are interconnected by straight lines. PS shows a marked tendency of flow dependence at flows below some 11-12 ml/min \times 100 g. In contrast, CFC is largely independent of flow in this state of maximal vasodilatation.

mean PS during control was in 25 experiments 5.67 ± 0.13 ml/min \times 100 g. In every experiment at least 6 control determinations of PS were performed.

3 Apparent PS versus rate of filtration-absorption

Whenever net filtration or absorption occurs, transcapillary solute transfer will to some extent be modified as shown in this study. The solute transfer could therefore be regarded as composed of a "pure" diffusion term (PS) and a convection term and they may together be called "apparent PS" (PS'). Concerning the present interaction between diffusion and filtration-absorption it should be stressed that only the blood to tissue diffusion transfer was dealt with because of the technique employed for diffusion studies. As briefly discussed in the Introduction this is not necessarily equivalent with the reversed transfer of O_2 produced metabolites or of a tracer that is uniformly dispersed in the interstitial space, which in e.g. skeletal muscle is narrow and with considerable intercapillary distances. The changes in extraction and in apparent PS were plotted against the rate of filtration-absorption as shown in Fig. 4. PS calculated from the modified Renkin-Crone formula, derived from the condition when perfusate inflow and outflow differed (see Methods), showed a slight but significant increase with increasing filtration and also a tiny decrease with increasing absorption. The slope of the linear regression of percentual increase in apparent PS on filtration rate was $9.47 \pm 2.18\%$ ml/min \times 100 g ($r=0.66$) and the slope of the percentual decrease in apparent PS on absorption rate was $4.28 \pm 1.81\%$ ml/min \times 100 g ($r=0.48$). The ordinate intercept was $1.78 \pm 1.30\%$ and $2.51 \pm 1.46\%$, respectively.

In accordance with the formulation by Kedem and Katchalsky (1958) the transfer of solute during filtration might be expressed as

$$PS = J_s - PS_d + J_v \frac{C}{2} (1 - \sigma)$$

$$\frac{P_{\text{net}}}{P_{\text{total}}} = 1$$

100-

APR 1970

100-

100
50

100

300

400

 $\frac{CFC_{\text{net}}}{CFC_{\text{total}}} \%$

The relative changes in PS on histamine infusion as the simultaneously recorded relative changes in CFC. The dotted lines show the theoretical relationship between the parameters at A) an isolated increase in radius but not in number of the small pores ($r \approx 40-60 \text{ \AA}$), B) an increase in radius but not in number of large pores ($r \approx 230 \text{ \AA}$, Groot 1956), C) an increase in number of the relatively few large pores, assuming a large to small pore number ratio of 1:30 000 as in 1956.

led by papaverine, the PS changes could be quite marked upon even moderate smooth muscle adjustments and consequent changes of flow resistance.

In contrast to the considerable changes in PS on even modest changes in the resistance to flow as a result of microvascular plugging, CFC always changed less. The observation that CFC changes are usually less extensive than the resistance changes on μ metabolic vasodilatation is evident also from earlier studies (e.g. Cobbold *et al.* 1963, Kjekshus 1964), and the present relationship between CFC and resistance closely resembles that presented by Kjekshus 1964 in connection with increasing metabolic vasodilatation in cat muscle.

Capillary diffusion and filtration capacity at histamine-induced permeability changes

An initially biogravitometric hindquarter preparation responded to histamine infusion (5-40 μg of perfusate) with often marked increases in CFC and in net transcapillary fluid filtration, causing weight increases of the preparation. The maximal CFC values recorded were some 0.20 ml/min mmHg 100 g, compared with around 0.04 ml/min mmHg 100 g at complete vasodilatation without histamine, and the maximal filtration rates were around 1.8 ml/min mmHg 100 g. These maximal permeability increases to histamine occurred at doses around 20 $\mu\text{g}/\text{ml}$ perfusate. Depending on the quality of the bovine serum, and together with the dextran as perfusate colloids, there was relatively great variations in

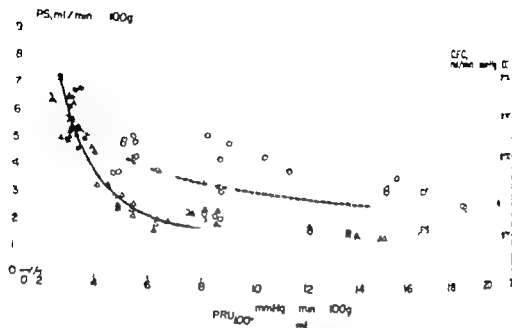


Fig. 5 CFC and PS-Cr-EDTA versus peripheral resistance at different levels of capillary plugging (microspheres $\phi = 15 \mu\text{m}$) during constant flow and maximal dilatation. Control values are CFC 4.6 ± 0.001 (SE) (ml/min mmHg $\times 100$ g) and PS 5.67 ± 0.13 (ml/min $\times 100$ g). The relationships would be the dotted line if the decrease in number of perfused capillaries were proportional to the increase in peripheral resistance during plugging. — PS seemed to change as theoretically predicted, whereas CFC changed less. Filled symbols, control values. Open symbols test values.

Methods) and the denominator the uncorrected E value according to the original Renkin-Crone formula (e.g. Renkin 1959). Uncorrected E value was 0.363 as PS was set at 5.67 Q at 12.5. For the matter of simplicity the solvent drag component of PS was set at zero, i.e. PS was held constant. Even if PS is allowed to change during correction, the relative change in E by concentration/dilution effects by convection is with a very good approximation, the same as that shown in Fig. 4.

4. Relationships between capillary diffusion and filtration capacities at alterations in capillary surface area

In Fig. 5 PS and CFC values are plotted against resistance to flow at maximal vasodilatation and at constant flow (around 12–13 ml/min 100 g) first, during the control situation and second, at different levels of rarification of the capillary bed by means of i.a. injected microspheres. The dotted line represents the expected decrease in both parameters in case the reduction in capillary surface area were exactly proportional to the increase in peripheral resistance. This would be the case only if vascular dimensions were not affected by the pressure increase secondary to microvascular plugging at constant flow and if this plugging excluded identical proportions of resistance and exchange vessels, i.e. if there were no anastomoses within the exchange network. On the whole, the PS changes seemed to follow the theoretical line relatively closely. Likewise, during control and before the vessels were fully

lar beds, which is practically impossible when complete uniformity is not at hand, but to study the relative changes in 'apparent' PS in different standardized situations, using in a model fashion principally important changes in microvascular function. To reduce flow heterogeneity to a minimum during control, the capillary bed was maximally dilated with papaverine, stagnant flow was avoided by using cell free perfusate and flow kept reasonably high, i.e. between 11 and 14 ml/min 100 g which is 3 times higher than resting steady state for muscle.

parent PS for Cr-EDTA increased with increasing flows and this tendency was most marked at flow below some 10 ml/min 100 g. This phenomenon, frequently observed (e.g. Renkin 1959, Yipintsoi *et al.* 1970, Trap-Jensen and Lassen 1970, Linde, Christensen and Rosell 1974, Pasaake 1977), here occurred even though the vascular bed was maximally dilated as checked by measurements of CFC. Thus, the flow-dependent increase in parent PS in this study cannot be explained by any increase in perfused capillary surface area. In other studies where capillary surface area was not controlled, recruitment of capillaries may of course, partly explain the PS dependence of flow (cf. Pasaake 1977). Other reasons behind this phenomenon is probably increasing heterogeneity of microvascular flow as perfusion pressure and flow decreases to low levels, resulting in semistagnant zones in some per se open loops which therefore hardly at all contribute to tracer transport. Further the chances for an increasing contribution of early back diffusion to the determinations of the extraction fraction may for the same reasons increase at low flows. However the impact of such factors on the PS determinations were here minimized when effects of changes in surface area and permeability were tested, because flow was then kept well above the level of substantial flow limitation for diffusion, as mentioned earlier.

The question whether convection is important or not as a mechanism for the blood to tissue transfer of small hydrophilic molecules has been a matter of some controversy over the last few decades. Transcapillary convection implies a direct transport of solute accompanying the flow of solvent through the capillary pores, i.e. solvent drag. Also, convection might indirectly increase the flux of solute through the membrane by e.g. improving mixing of the interstitial fluid, and thus preventing interstitial gradients to build up. By this mechanism the interstitial concentration would be lowered and diffusion across the capillary wall augmented according to Fick's law. In addition, the transmembrane diffusion coefficients might be changed also by concentration/dilution effects which are produced when the process is reflected to a greater extent by the microvascular membrane than water during reabsorption.

With the single injection technique it is possible to study the solvent drag effects and the concentration/dilution effects on solute transfer. In this study correction was made for the impact of the last-mentioned factor by means of estimating solute transport from a modified Renkin-Crone formula, which takes into consideration convection-induced changes in flow through the system (see Methods). However in this blood to tissue transfer method starting effects would not cause changes in extraction, at least not under ideal conditions, because the extraction determination is made from the first few seconds of the single injection curves (around peak time) when tissue concentration is supposed to be close

the extent of maximal histamine response among individual experiments. Quite unexpected the greatest shifts in permeability by histamine were obtained if "aged" horse serum (stored for 3 weeks at $+4^{\circ}\text{C}$) was used in the perfusate. This is probably due to the fact that old horse serum contains functional histamine antagonists with respect to capillary permeability, such as catecholamines, as dealt with in another study (Rippe and Grega 1978).

In contrast to the gross changes in capillary fluid equilibrium and CFC induced by histamine, PS for Cr EDTA changed very little. The range of PS increment was of the order of 0–20% from control as shown in Fig. 6. It is, however, important to realize that the total transport in these experiments is somewhat underestimated because of histamine induced leakage of the nonpermeant tracer. The error of not taking this factor into account results in an underestimation of solute transfer of 7–15%, as discussed under Methods. Therefore the maximal change in total transport of Cr EDTA after histamine is some 20–30% of which about two thirds is the result of increased diffusion. This is to be compared with the observed increase in CFC of maximally 400–500%.

Discussion

The single injection indicator diffusion method has been used extensively in studies of unidirectional blood to tissue transfer of various molecules (e.g. Crooke and Lassen 1970; Bassingthwaite 1974), but only seldom has it been possible to combine it with other methods for functional capillary studies. Therefore the present study was performed, combining this method with the isogravimetric technique (Pappenheimer and Soto 1948) to allow simultaneous measurements of diffusion and filtration in the rat hindlimb vascular bed during controlled artificial perfusion when pressure, flow, pre- and postcapillary resistances, capillary surface area, capillary permeability etc. could be more or less independently changed to a known extent.

From most earlier single injection studies it is evident that the curve representing the fractional extraction versus time ($E(t)$) is highly variable in shape depending on the tissue studied, the sampling system employed, flow rate, etc. In the present study the most common shape of the $E(t)$ curve was a rapid initial increase followed by a biphasic decrease and a tracer curve peak time, by a plateau lasting for some seconds to again decrease gradually. The most likely explanation for the initial peak in $E(t)$ is Taylor diffusion (cf. Taylor 1954) while the most probable mechanism behind the second slower rise in $E(t)$ preceding the plateau is heterogeneity in capillary arrangement and hence in flow. Heterogeneity in this sense is however a normal feature of all capillary networks, even in the relatively uniform myocardial vascular bed where rising $E(t)$ curves have also been registered (e.g. Downes and Kirk 1970; Bassingthwaite, Yipintsoi and Grabowski 1975). The presence of such heterogeneity inherent in the very arrangement of capillary networks and supply routes implies that when out-diffusion starts in some capillaries, back diffusion may have already started in others. These normal consequences of how vascular beds are designed tend, however, to reduce the experimentally recorded extraction fraction and will thus underestimate the real permeability surface area (PS) product. The aim of the present study was, however, not to give an absolutely precise estimation of the "true" PS product for Cr EDTA in excised

or beds, which is practically impossible when complete uniformity is not at hand, but to study the relative changes in apparent PS in different standardized situations, using in a model fashion principally important changes in microvascular function. To flow heterogeneity to a minimum during control, the capillary bed was maximally perfused with papaverine, stagnant flow was avoided by using cell free perfusate and flow rate reasonably high, f between 11 and 14 ml/min 100 g which is 2-3 times higher than resting steady state for muscle.

Apparent PS for Cr-EDTA increased with increasing flows and this tendency was most marked at flows below some 10 ml/min 100 g. This phenomenon, frequently observed (e.g. Renkin 1959, Yipintsoi *et al.* 1970, Trap-Jensen and Lassen 1970, Linde, Christensen and Rowell 1974, Paaske 1977), here occurred even though the vascular bed was maximally dilated as checked by measurements of CFC. Thus the flow-dependent increase in apparent PS in this study cannot be explained by any increase in perfused capillary surface area. In other studies where capillary surface area was not controlled, recruitment of capillaries may of course partly explain the PS dependence of flow (cf. Paaske 1977). Other reasons behind this phenomenon is probably increasing heterogeneity of microvascular flow when perfusion pressure and flow decreases to low levels, resulting in semistagnant flow in some per se open loops which therefore hardly at all contribute to tracer transport. Further the chances for an increasing contribution of early back diffusion to the determinations of the extraction fraction may for the same reasons increase at low flows. However the impact of such factors on the PS determinations were here minimized when the effects of changes in surface area and permeability were tested, because flow was then kept well above the level of substantial flow limitation for diffusion, as mentioned earlier.

The question whether convection is important or not as a mechanism for the blood to tissue transfer of small hydrophilic molecules has been a matter of some controversy over the last few decades. Transcapillary convection implies a direct transport of solute accompanying the flow of solvent through the capillary pores, i.e. solvent drag. Also, convection might indirectly increase the flux of solute through the membrane by e.g. improving mixing of the interstitial fluid, and thus preventing interstitial gradients to build up. By this mechanism the interstitial concentration would be lowered and diffusion across the capillary wall augmented according to Fick's law. In addition, the transmembrane diffusion coefficients might be changed also by concentration/dilution effects which are produced when water is reflected to a greater extent by the microvascular membrane than water during solute absorption.

With the single injection technique it is possible to study the solvent drag effects and the low concentration/dilution effects on solute transfer. In this study correction was made for the impact of the last-mentioned factor by means of estimating solute transport from a modified Renkin-Crone formula, which takes into consideration convection-induced changes in flow through the system (see Methods). However in this blood to tissue transfer model interstitial stirring effects would not cause changes in extraction, at least not under ideal conditions, because the extraction determination is made from the first few seconds of the single injection curves (around peak time) when tissue concentration is supposed to be close

to zero. Therefore, to study interstitial stirring effects it is far more rational to follow the diffusion from the interstitium to blood with other techniques (*cf.* Lundgren and Mörner 1967) or by using the 'tail' of the single injection curves when the indicator returns from the tissue spaces to the blood stream (Paaske 1977, Kamiya, Rippe and Folkow unpublished results). Another possibility to study stirring effects would be to use the constant infusion technique by which it might be possible to assess both capillary membrane, interstitial diffusion and cell membrane impedances (Sheehan and Reiss 1977). The conclusion that might be drawn from the present data is that convection from blood to tissue transfer of solute to an extent that can be theoretically predicted from the theory of irreversible thermodynamics (Kedem and Katchalsky 1958). This is in agreement with the results from recent single injection measurements on the blood perfused, microvasodilated dog hindlimb (Fleming and Diana 1976). With the present data it is, however, not possible to give an accurate estimation of the reflection coefficient of Cr-EDTA.

In essence, the present results imply that convection is normally not a quantitatively important mechanism for the transport of small molecular weight solutes across the capillary membrane *per se*. Even at heavy muscular exercise, when the filtration rate might reach to 0.5–1 ml/min/100 g of tissue, the contribution of filtration to the transcapillary transport of small molecules from blood to tissue is below 10% of the total transport obtained for Cr-EDTA (Mw 341). This convection contribution to transport is theoretically still lower for molecules smaller than Cr-EDTA.

Upon reducing the capillary surface area by microvascular plugging PS for Cr-EDTA, as measured during constant flow and maximal vasodilatation, was markedly reduced and the reductions of CFC were always relatively smaller. Both relatively and absolutely the PS reduction was most pronounced at changes in resistance to flow from 2.8 to about 8 PRU (ml/min/100 g), these resistance figures being valid for a perfusate viscosity slightly above two thirds of that for blood *in vivo*. Fig. 4 shows the asymptotic description of the theoretical relation between PS and resistance to flow. If vascular dimensions were not affected by the transmural pressure increase produced by microvascular plugging and if there were no collateral interconnections between this level and the exchange vasculature. The equation of this theoretical function is $(PS/5.67) = (2.8/R)$ where R is the peripheral resistance per 100 g of tissue (PRU) and 2.8 is the PRU at maximal vasodilatation when PS is 5.67 ml/min/100 g. The vascular distension occurring when resistance and hence pressure is increased as a result of plugging, tends to lower the PS vs. R relation below the theoretical line. Further there are probably some collateral interconnections between the plugging sites and the exchange vessels by which relatively more capillaries can be recruited particularly at higher pressures, giving higher PS values at high PRU values than predicted by the theoretical curve. In addition, solute transfer studied with the dual indicator perfusion method might also be affected by the fact that heterogeneity in microvascular perfusion increases with plugging, whereas the chances of early back diffusion decrease.

When comparing the present PS data with those for Cr-EDTA (Mw 341) or more generally for other species variations must be taken into account. At maximal vasodilatation PS for Cr-EDTA would be some 5.5–6 ml/min/100 g in rat, cat and dog muscle vasculature (Paaske 1977).

ing and Dunn 1976) and in the resting state around 1.5 ml/min 100 g (recalculated from Croce 1963 on cat, assuming a resting plasma flow of 4 ml/min 100 g), while at sublethal flows during work hyperemia the values would fall inbetween (Trap-Jensen and Jørgensen 1970, man's forearm). Assuming a capillary surface area of 7000 cm² (cf Landis and Pappenheimer 1963) at maximal vasodilatation a permeability value (P_d) for sucrose or DTA of some $1.4-1.5 \cdot 10^{-8}$ cm/s is obtained.

Concerning diffusion exchange the relative shift of perfused capillary surface area in skeletal muscle from rest to maximal vasodilatation during heavy exercise would thus be about 5 times (Fig. 5). However when estimated from CFC determinations this figure is only around 3 times, according to Fig. 5. The capillary surface area available for *diffusion* exchange in the resting state thus seems to be overestimated when judged by CFC measurements. The main reason seems to be that during a CFC determination the capillary pressure also transmits also to capillaries where flow is too low to be of significant relevance for *diffusion* exchange, but where the far less flow-limited filtration process still can take place (cf Folkow and Mellander 1970). For such reasons, PS estimations provide the most exact picture of the number of capillaries available for effective *diffusion* exchange of solutes, while CFC measurements as precisely reflect the capillary surface available for *fluid* exchange between intra- and extravascular spaces, which for *plasma* volume dilution is the relevant thing. These two types of measurements thus reflect two biologically important but different aspects of the transcapillary exchange processes. When these processes are earlier measured separately marked changes in diffusion capacity as a response to reduction in capillary surface area have usually been observed (e.g. Renkin and Rosell 1958). However the changes in CFC on e.g. metabolic vasodilatation in skeletal muscle have usually been observed to be relatively smaller (cf Cobbold *et al.* 1963 Kjellmer 1964), which is in agreement with the present measurements of both processes simultaneously. Histamine infused into the hindquarter vascular bed markedly increased CFC and produced net fluid filtration in the initially isogravimetric preparation, but did not alter vascular resistance in the already maximally vasodilated preparation. This marked fluid filtration must therefore be due to a decrease in effective transcapillary colloid osmotic pressure difference or in other words, to marked decrease of the reflection coefficient for the perfusate macromolecules (dextran, 70 000, and albumin). In this situation histamine produced a maximal filtration rate of 1.7-1.8 ml/min 100 g and CFC increased from around 0.04 to maximum 0.2 ml/min mmHg 100 g. Dividing 1.7 by 0.2 gives the maximal decrease in effective colloid osmotic pressure produced by histamine in these experiments, i.e. 8.5 mmHg.

The present relative changes in both CFC and isogravimetric capillary pressure (P_{nc}) are lower than those reported by McNamee and Grodins (1975) probably because the present measurements set out from the maximally vasodilated state where the greater number of perfused capillaries implies that CFC is around three times higher than in the resting steady state. It cannot be ascribed to any initially abnormal permeability increase to water or small molecules in the present preparation, as judged from the CFC and PS data. However the permeability to macromolecules is somewhat increased during dextran perfusion by unknown mechanisms (Areekul 1969 Rippe and Folkow 1977), and this may be the

to zero. Therefore to study interstitial stirring effects it is far more rational to follow diffusion from the interstitium to blood with other techniques (*cf.* Lundgren and McFate 1967) or by using the 'tail' of the single injection curves when the indicator moves out from the tissue spaces to the blood stream (Paaske 1977, Kamiya, Rippe and Folkow unpublished results). Another possibility to study stirring effects would be to use the constant infusion technique by which it might be possible to assess both capillary membrane, interstitial diffusion and cell membrane impedances (Sheehan and Reiss 1977). The conclusion that might be drawn from the present data is that convection mediates blood to tissue transfer of solute to an extent that can be theoretically predicted from a theory of irreversible thermodynamics (Kedem and Katchalsky 1958). This is in agreement with the results from recent single injection measurements on the blood perfused, anesthetized, vasodilated dog hindlimb (Fleming and Diana 1976). With the present data it is, however, not possible to give an accurate estimation of the reflection coefficient of Cr-EDTA.

In essence the present results imply that convection is normally not a quantitatively important mechanism for the transport of small molecular weight solutes across the capillary membrane *per se*. Even at heavy muscular exercise, when the filtration rate is about 0.5–1 ml/min 100 g of tissue, the contribution of filtration to the transcapillary transfer of small molecules from blood to tissue is below 10% of the total transport obtained with Cr-EDTA (Mw 341). This convection contribution to transport is theoretically still valid for molecules smaller than Cr-EDTA.

Upon reducing the capillary surface area by microvascular plugging PS for Cr-EDTA, as measured during constant flow and maximal vasodilatation, was markedly reduced but the reductions of CFC were always relatively smaller. Both relatively and absolutely the PS reduction was most pronounced at changes in resistance to flow from 2.8 to 8 PRU ∞ (mmHg ml⁻¹ min⁻¹ 100 g), these resistance figures being valid for a perfusate viscosity slightly above two thirds of that for blood *in vivo*. Fig. 4 shows the asymptotic description of the theoretical relation between PS and resistance to flow if vascular dimensions were not affected by the transmural pressure increase produced by microvascular plugging and if there were no collateral interconnections between this level and the exchange vasculature. The equation of this theoretical function is $(PS/5.67) = (2.8/R)$ where R is the peripheral resistance per 100 g of tissue (PRU ∞) and 2.8 is the PRU ∞ at maximal vasodilatation where PS is 5.67 ml/min 100 g. The vascular distension occurring when resistance and perfusion pressure is increased as a result of plugging, tends to lower the PS vs. R relation below the theoretical line. Further there are probably some collateral interconnections between the plugging sites and the exchange vessels by which relatively more capillaries can be recruited, particularly at higher pressures, giving higher PS values at high PRU ∞ values than predicted by the theoretical curve. In addition solute transfer studied with the dual indicator single injection method might also be affected by the fact that heterogeneity in microvascular perfusion increases with plugging, whereas the chances of early back diffusion decrease.

When comparing the present PS data with those for Cr-EDTA (Mw 341) or sucrose (Mw 342) obtained in other laboratories the hemodynamic state of the preparation as well as species variations must be taken into account. At maximal vasodilatation PS for Cr-EDTA would be some 5.5–6 ml/min 100 g in rat, cat and dog muscle vasculature (Paaske 1977).

$$PS_{\text{hist}}/PS_{\text{control}} - 100 \left(\frac{r}{r_1} \right) (CFC_{\text{hist}}/CFC_{\text{control}} - 100)$$

For all parameters except (r_0/r_1) , which is the ratio of radii of small to large pores, are defined in the Figure. (See appendix for derivation.) From this equation it is evident that for $r = 40$ and $r_1 = 250$ (Grotte 1936), a fourfold CFC increase which would occur if the number of large pores increased 60 times, assuming one large pore in 30 000 small pores in control situation, would increase PS only 7.7%. Both large pore and small pore radii are today often considered to be somewhat larger (e.g. Winne 1965 Paaske 1977) than the values used in the calculation above. Therefore, the r_0/r_1 value used in Figure 6 may be too small and a lower value will shift the relation towards the abscissa. The line II in Figure 6 is described by the following equation, as derived in the appendix.

$$PS_{\text{hist}}/PS_{\text{control}} - 100 \sqrt{100(CFC_{\text{hist}}/CFC_{\text{control}} - 100) \frac{n_1}{n_0}}$$

where n_1/n_0 is the ratio of number of large to small pores. It describes the relation of relative PS and CFC changes when the radii of the large pores are uniformly increased, while the small pore radii remain constant. At a CFC increase up to 400%, $PS_{\text{hist}}/PS_{\text{control}}$ would increase only by 1%, using a value of n_1/n_0 of 1/30 000 for the calculations. A still smaller n_1/n_0 ratio (cf. Winne 1965 Renkin, Carter and Joyner 1974) would shift also this relation towards the abscissa.

Line C in Fig. 6 can be reasonably well fitted to the present experimental data and the conclusion would be that the histamine effect on the microvascular membrane in principle implies an opening of additional large pores, increasing their number fifty to hundredfold. This agrees well with morphological *in vitro* data (e.g. Majno and Palade 1961), and with *in vivo* observations (e.g. Grega, personal communication). The data from the present study are, however, entirely contradictory to recent suggestions (Renkin, Carter and Joyner 1974, Carter, Joyner and Renkin 1974) that histamine should enhance vesicular transport rather than increasing the number of large pores. If so, CFC should remain largely unchanged while PS for Cr-EDTA would increase to some extent by histamine, implying that the relation between relative PS and CFC would shift to the other extreme in Fig. 6, i.e. towards the ordinate axis.

To summarize, it was in the present study possible to follow diffusion and filtration events with highly reproducible, simultaneous measurements in a well controlled, constant-flow perfused model preparation over a wide range of induced changes in capillary surface area, permeability and fluid filtration. Hopefully the data from this study have shown that further investigation of capillary membrane characteristics in other vascular beds and tissues by such an approach with combined techniques is needed for a full understanding of the mechanisms behind the transcapillary exchange of molecules.

Appendix

Derivation of the equations for lines C and II in Fig. 6:

The values obtained during histamine infusion on $PS_{\text{hist}}/PS_{\text{control}}$ (—) were plotted against the similarly obtained values on $CFC_{\text{hist}}/CFC_{\text{control}}$ () as shown in Fig. 6. According to the double pore

reason why the present P_{ci} decrease was slightly lower than that obtained by McNamee and Grodins

P_{ci} for this dextran-horse serum—Tyrode perfused preparation has earlier been determined to 12.4 mmHg (Kamiya, Rippe and Folkow 1978) and the reflection coefficient σ for the perfusate colloids is then around 0.5 (Areekul 1969; Rippe and Folkow 1977). According to the modified Starling theory the following is valid

$$P_{ci} = P_i + \sigma(\pi - \pi_i)$$

where P_{ci} and σ were defined above and P_i is the interstitial fluid pressure, while π and π_i are the colloid osmotic pressures in the perfusate and the interstitium, respectively. For the sake of simplicity P_i could be set to zero (cf. Eliassen *et al.* 1974). Knowing the P_{ci} value before and after histamine and control σ a rough estimation of σ for the perfusate colloid during histamine is obtained if P_i and π_i are assumed to remain largely constant, which would approximately hold for the first few minutes of histamine infusion. σ for the perfusate macromolecules during histamine infusion would then be around 0.15 and had thus been reduced markedly from control when it was around 0.5.

In contrast to the pronounced changes in CFC and macromolecular permeability during histamine infusion, PS for Cr EDTA changed very little (cf. Rippe and Grega 1978). This is in principal agreement with findings by *e.g.* Diana, Long and Yao (1972) although the changes also in CFC and P_{ci} were much smaller and more transient. In the great majority of experiments on canine adipose tissue Linde, Chisholm and Rosell (1974) noted only small changes in PS for sucrose on histamine infusion, around 25% increase. Further failure to show substantial effects of histamine on small molecular permeability has been experienced by other investigators (Lassen, personal communication).

This discrepancy between the marked histamine-induced changes in hydraulic conductivity and large molecular permeability on the one hand, and the almost insignificant increase in small molecular permeability on the other can best be explained by a preferential action of histamine on venular gap formations (large pores), increasing their number or just their radii as illustrated in Fig. 6. This Figure also illustrates the theoretical relations between the two parameters in four different situations: A. At an increase in the number of small pores; B. At a uniform increase in small pore radius; C. At a conversion of a minor fraction of small pores to large pores ($r \sim 250$ Å) or simply opening more large pores; and D. At an increase in radius of preexisting large pores. Vesicular transport as a possible mechanism of solute transfer was not considered here, partly because the present data cannot be explained by this mechanism for reasons mentioned below, partly because more direct evidence against this mechanism for transcapillary macromolecular transfer was recently advanced (Rippe, Kamiya and Folkow 1977).

Line A assumes a linear increase in number of small pores with parallel increases in PS and CFC. Line B assumes a uniform small pore radius increase, increasing hydraulic conductivity to the fourth power of the radius (Poiseuille's law) and small molecular diffusion capacity (nonrestricted diffusion) to the second power of the radius (Fick's law). Line C is a variant of line A where the number but not the radius of large pores increases. The equation for this line is

$$PS_{\text{test}}/PS_{\text{control}} - 100 = \left(\frac{r_s}{r_l} \right)^2 (CFC_{\text{test}}/CFC_{\text{control}} - 100)$$

all parameters except (r_s/r_l) , which is the ratio of radii of small to large pores, are used in the Figure. (See appendix for derivation.) From this equation it is evident that for $r_s/r_l = 40$ and $r = 250$ (Grotte 1956), a fourfold CFC increase which would occur if the number of large pores increased 60 times, assuming one large pore in 30 000 small pores as control situation, would increase PS only 7.7%. Both large pore and small pore radii are often considered to be somewhat larger (e.g. Winne 1965 Pauske 1977) than the values used in the calculation above. Therefore, the r_s/r_l value used in Figure 6 may be too small and a lower value will shift the relation towards the abscissa. Line B in Figure 6 is described by the following equation, as derived in the appendix.

$$PS_{\text{test}}/PS_{\text{control}} - 100 = \sqrt{100(CFC_{\text{test}}/CFC_{\text{control}} - 100) \frac{n_l}{n_s}}$$

where n_l/n_s is the ratio of number of large to small pores. It describes the relation of relative PS and CFC changes when the radii of the large pores are uniformly increased, while the small pore radii remain constant. At a CFC increase up to 400%, $PS_{\text{test}}/PS_{\text{control}}$ would rise only by 1%, using a value of n_l/n_s of 1/30 000 for the calculations. A still smaller n_l/n_s ratio (cf. Winne 1965 Renkin, Carter and Joyner 1974) would shift also this relation towards the abscissa.

Line C in Fig. 6 can be reasonably well fitted to the present experimental data and the conclusion would be that the histamine effect on the microvascular membrane in principle implies an opening of additional large pores, increasing their number fifty to hundredfold. This agrees well with morphological *in vitro* data (e.g. Majno and Palade 1961), and with *in vivo* observations (Grega, personal communication). The data from the present study are, however, entirely contradictory to recent suggestions (Renkin, Carter and Joyner 1974, Carter, Joyner and Renkin 1974) that histamine should enhance vesicular transport rather than increasing the number of large pores. If so, CFC should remain largely unchanged while PS for Cr-EDTA would increase to some extent by histamine, implying that the relation between relative PS and CFC would shift to the other extreme in Fig. 6, i.e. towards the ordinate axis.

To summarize, it was in the present study possible to follow diffusion and filtration events with highly reproducible, simultaneous measurements in a well controlled, constant-flow perfused model preparation over a wide range of induced changes in capillary surface area, permeability and fluid filtration. Hopefully the data from this study have shown that further investigation of capillary membrane characteristics in other vascular beds and tissues by such an approach with combined techniques is needed for a full understanding of the mechanisms behind the transcapillary exchange of molecules.

Appendix

Derivation of the equations for lines C and D in Fig. 6.
The values obtained during histamine infusion on $PS_{\text{test}}/PS_{\text{control}}$ () are plotted against the simultaneously obtained values on $CFC_{\text{test}}/CFC_{\text{control}}$ () as shown in Fig. 6. According to the 'double pore'

theory (Grotte 1956) the percentual change in CFC_{test} and PS_{test} from control (100) during histamine infusion can be expressed according to Potts and Fick's laws, respectively (assuming non-restricted filtration/diffusion) as

$$\frac{CFC_{test}}{CFC_{control}} = 100 \frac{r_1 + n_2 r^4}{n_2 r} = 100 \left[\frac{n_1}{n_2} \left(\frac{r_1}{r} \right) + 1 \right]$$

$$\frac{PS_{test}}{PS_{control}} = 100 \frac{n_1 r_1^2 + n_2 r^2}{n_2 r^2} = 100 \left[\frac{n_1}{n_2} \left(\frac{r_1}{r} \right)^2 + 1 \right]$$

Here n_1/n_2 is the ratio of number of large to small pores, r_1/r the ratio between their radii. $CFC_{control}$ and $PS_{control}$ represent the PS and CFC values in the theoretical situation where large pore filtration and diffusion are zero. In reality CFC, and to some extent PS, during control are slightly larger than the theoretical control values because of a minor contribution of large pore filtration and diffusion to transport even before histamine infusion (Grotte 1956 see further below.) Eq. (1) and (2) give

$$\frac{PS_{test}}{PS_{control}} - 100 = \left(\frac{n_1}{n_2} \right)^2 \left(\frac{CFC_{test}}{CFC_{control}} - 100 \right)$$

Eq. (3) is the equation for line C in Fig. 6. Here n_1/n_2 is allowed to change while r_1/r is set constant. Changing n_1/n_2 from 1/30 000 (Grotte 1956) to 59/30 000 induces a change in $CFC_{test}/CFC_{control}$ from 100.1 to 400 according to Eq. (1), while $PS_{test}/PS_{control}$ increases from 100.1 to 107.7 according to Eq. (2) and (3) assuming a value on r/r_1 of 6.25 (Grotte 1956).

Rearranging Eq. (2) gives

$$r^2 = 100 \frac{n_1}{n_2} r_1^2 \left(\frac{PS_{test}}{PS_{control}} - 100 \right)$$

Substituting Eq. (4) in Eq. (3) gives

$$\left(\frac{PS_{test}}{PS_{control}} - 100 \right)^2 = 100 \frac{1}{\left(\frac{CFC_{test}}{CFC_{control}} - 100 \right)}$$

The positive square root of both sides of Eq. (5) gives the equation of line D in Fig. 6. Here n_1/n_2 is allowed to change while r_1/n_2 is set constant. Changing r_1/r from 6.25 (see above) to 17.32 induces a change in $CFC_{test}/CFC_{control}$ from 100.1 to 400 according to Eq. (1), while $PS_{test}/PS_{control}$ changes from 101.1 to 101.0 according to Eq. (2) and Eq. (5), assuming a value on n_1/n_2 of 1/30 000 (see above).

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On the capacity of the β -oxidation of palmitate and palmitoyl-esters in rat liver mitochondria

By

MICHAEL FARSTAD AND ROLF BERGE

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Abstract

STAD, M. and R. BERGE. *On the capacity of the β -oxidation of palmitate and palmitoyl-esters in rat liver mitochondria* Acta physiol. scand. 1978, 104, 337-348.

β -oxidation of palmitate, palmitoyl-CoA and palmitoyl-L-carnitine proceeded at high rate in isolated rat mitochondria. At high concentrations (100 μ mol/mg protein) the oxidation of palmitate and palmitoyl-CoA was only partly carnitine dependent. All substrates were most rapidly oxidized in the presence of succinate and state 3 conditions. Succinate inhibited β -oxidation especially in state 4 conditions. β -oxidation was faster in hypotonic than in isotonic medium both in state 3 and state 4 conditions. Hypercarnitine inhibited β -oxidation. The rapid formation of palmitoyl-CoA from palmitate, CoA and ATP faster than the oxidation of palmitate under identical conditions. The presence of bovine serum albumin slowed the β -oxidation, especially with palmitoyl-CoA or free palmitate as the substrates. Mitochondria lacking palmitoyl-CoA hydrolase back may influence the available extramitochondrial palmitoyl-CoA. Present results demonstrate no single rate limiting step in the β -oxidation *in vivo*. Both the NADH/D ratio, competition for the respiratory chain, the level of ADP binding of palmitoyl-CoA to extramitochondrial protein, and possibly intramitochondrial hydrolysis of palmitoyl-CoA all seem to influence rate of β -oxidation *in vivo*. It is suggested that *in vivo* the most important factor is the availability of CoA to the other carnitine palmitoyl-transferase of the mitochondria.

β -oxidation of fatty acids includes the formation of acyl-CoA, transfer of the acyl-group to carnitine with subsequent translocation of the acyl-carnitine through the inner mitochondrial membrane, acylation of intramitochondrial CoA, entrance of acyl-CoA into the oxidation cycle and transfer of the electrons through the electron transport chain. Any of these steps could be rate-limiting, either by the activity of the involved enzymes, by the availability of competing substrates or by competition for cofactors such as CoA or NAD. Previously published results have indicated that the fatty acid activation (Schultz *et al.* 1972, Fitzmark and Pedersen 1975, Oram *et al.* 1973), the synthesis or translocation of L-carnitine, (Oram *et al.* 1973, Shephard *et al.* 1966, Fritz 1968, McGarry *et al.* 1973) or capacity of the β -oxidation cycle or the capacity of the electron transport chain (Pande 1971) might be the rate limiting step. Several of the involved reactions may vary under different experimental or metabolic conditions in different organ systems from various

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β -Oxidation of fatty acids includes the formation of acyl-CoA, transfer of the acyl-group to carnitine with subsequent translocation of the acyl-carnitine through the inner mitochondrial membrane, acylation of intramitochondrial CoA, entrance of acyl-CoA into the β -oxidation cycle and transfer of the electrons through the electron transport chain. Any of these steps could be rate-limiting, either by the activity of the involved enzymes, by the availability of competing substrates or by competition for cofactors such as CoA or NAD. Previously published results have indicated that the fatty acid activation (Schultz *et al.* 1972, Flück *et al.* 1973, Orum *et al.* 1973), the synthesis or translocation of β -carnitine (Orum *et al.* 1973, Shephard *et al.* 1966, Fritz 1968, McGarry *et al.* 1973) or the capacity of the β -oxidation cycle or the capacity of the electron transport chain (Pande 1971) might be the rate limiting step. Several of the involved reactions may vary under different experimental- or metabolic conditions in different organ systems from various

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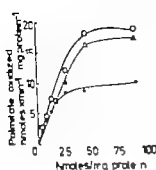


Fig. 1

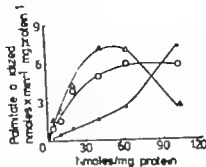


Fig. 2

1. The formation of ^{14}C -acetyl-groups from ^{14}C -palmitoyl-L-carnitine as a function of the concentration of palmitoyl-L-carnitine. The incubation system contained in final volume of 300 μl 100 mM ES-buffer pH 7.2, 4 mM dithiothreitol and 2.5 mM MgCl_2 . Amount of mitochondrial proteins was 1.2 μg . Incubation temperature 35 $^\circ\text{C}$ and incubation time 2 min. ^{14}C -palmitoyl-L-carnitine was added as 1 μM . Reaction was stopped by 150 μl 1.5 M KOH and BSA as added to concentration of 5 mg/ml. After precipitation, 300 μl 4 M HClO_4 was added, and after precipitation of the insoluble substrate, an aliquot of the supernatant was transferred to counting vial containing 5 ml Unisolve H_2O (see addition). \circ — \circ State 4 (1 mM ADP, 1 mM phosphate); \triangle — \triangle State 3 (4 μM CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone)).

2. The carnitine dependent formation of ^{14}C -acetyl-groups from ^{14}C -palmitoyl-CoA as a function of the concentration of palmitoyl-CoA in the presence of 20 mM carnitine. State 3 conditions are used by addition of 1 mM ADP, 1 mM phosphate. Conditions otherwise are as stated in Legend. \circ — \circ Absence of carnitine (no carnitine added); \circ — \circ State 4 (presence of carnitine—absence of carnitine); \triangle — \triangle State 3 (presence of carnitine—absence of carnitine).

Incubation was as stated in the Tables and Legends. Figures. Aliquot of the perchloric acid-soluble fraction were counted in 5 ml Unisolve.

3. Formation of ^{14}C -palmitoyl-CoA from ^{14}C -palmitate, CoA and ATP as assayed as indicated in Fig. 2, and ^{14}C -palmitoyl-CoA formed as extracted according to Bar-Tana *et al.* (1971).

4. Palmitoyl-CoA hydrolysis as assayed by the release of ^{14}C -palmitic acid from ^{14}C -palmitoyl-CoA as described (Berge and Farnstedt 1973).

5. Respiratory control of the mitochondria (R.C._{ADP}) as checked by the oxygen uptake with succinate substrate in the absence and presence of ADP using Clark oxygen electrode (Radco-meter, Copenhagen, Denmark) (Ottav *et al.* 1970).

Results

Formation of ^{14}CO from ^{14}C -labelled substrates

Under the described conditions the formation of ^{14}CO was low during the first 1–2 min, then as linear with time up to about 6 min with all substrates (results not shown). In all experiments, the production of CO_2 was less than 4 per cent of the total acetyl-group formation provided the incubation time was 2 min or less. Thus, the error introduced by neglecting the CO formation probably was within the over-all experimental error.

Formation of ^{14}C -acetyl-groups from ^{14}C -labelled palmitoyl-L-carnitine, palmitoyl-CoA and palmitic acid

Fig. 1 shows the formation of ^{14}C -acetyl-groups from ^{14}C -palmitoyl-L-carnitine as a function of the palmitoyl-L-carnitine concentration. Saturation concentration was about 50 nmol/mg protein under state 4 conditions. A half saturation concentration, or an

species (Oram *et al* 1973, Pande 1971, Bremer and Wojtczak 1972, Lurberg *et al* 1974). Fasting has been reported to have significant influence on fatty acid oxidation, although to a variable extent from species to species (Sblin *et al* 1970), or no significant influence (D'Alecco and Hoppel 1975).

Also an intramitochondrial long-chain acyl-CoA hydrolase recently described (Berge and Farstad 1978) is sufficiently active to influence the intramitochondrial acyl-CoA available for the β -oxidation cycle.

The formation of acid soluble ^{14}C labelled oxidation products from ^{14}C -palmitoyl-L-carnitine has been shown to be a reliable method for the study of the β -oxidation (Larsson *et al* 1970, Bremer and Wojtczak 1972, Oram *et al* 1973). The present communication is a study of factors that influence β -oxidation *in vitro*.

Materials and methods

Reagents

1 ^{14}C palmitoyl-L-carnitine (spec. act. 54 mCi/mmol), 1- ^{14}C -palmitoyl-CoA (spec. act. 56 mCi/mmol) and 1 ^{14}C -palmitic acid (spec. act. 52 mCi/mmol) were purchased from New England Nuclear, Boston, Ma. U.S.A., palmitoyl-L-carnitine and L-carnitine from Supelco Inc., Bellefonte, Pa. U.S.A. and other chemicals from Sigma Chemical Company, St. Louis, Mo. U.S.A.

1 ^{14}C -labelled palmitoyl-carnitine and palmitoyl-CoA were dissolved in 30 per cent ethanol to a concentration of 5 and 0.5 mM respectively. Palmitate was used as a 5 mM aqueous solution of the potassium salt. The specific activities of all substrates were about 500 000 counts/min per pmole. Incubation in 5 ml Unisol C (Koch Light, Colbrook, Bucks, England) in a Packard Tri-Carb Scintillation Counter Model 2450.

Preparation of mitochondria

Male albino rats (Wistar-Møll) of 150–200 g were killed and bled after fasting overnight. The livers were immediately cut in pieces, cooled in ice and the mitochondria were isolated as described by Randle and Flatmark (1973). Protein was determined by a modified Lowry procedure (Flatmark *et al* 1971). The final mitochondrial suspensions contained about 50 mg protein per ml.

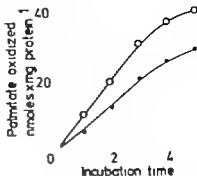
Preparation of mitochondrial matrix protein

Isolated mitochondria were treated with 0.12 mg digitonin per mg mitochondrial protein, and the mitochondrial matrix was isolated by centrifugation of the isolated mitoplasts as described by Berge and Farstad (1978). The isolated matrix protein was virtually free from lysosomal and microsomal enzymes (Berge and Farstad 1978).

Assay methods

Production of $^{14}\text{CO}_2$ from ^{14}C -labelled substrates was assayed in 100 mM N-2-hydroxyethyl-piperazine ethane-sulfonic acid (HEPES) pH 7.2 in Warburg flasks with 100 μl 1 M KOH in the center cell and 1 μl 1 M H_2SO_4 in the side arm. The incubation medium contained 200 μmol of the radioactive substrate and 5 mg crystalline bovine serum albumin (BSA) per ml (about 40 $\mu\text{mol}/\text{mg}$ protein). Incubation temperature was 35°C.

The acid-soluble radioactivity from ^{14}C -labelled palmitoyl-L-carnitine is a mixture of ketone bodies and Krebs cycle intermediates (LaNoue *et al* 1970), and thus is a measure of the β -oxidation through the formation of acetyl-groups. The standard incubation for the formation of 1 ^{14}C -acetyl-groups from 1 ^{14}C -labelled substrates, which is a measure of palmitoyl-group utilization, was performed according to Bremer and Wojtczak (1972) with some modifications. 40–50 μmol of 1 ^{14}C -palmitoyl-CoA or palmitoyl-L-carnitine and 70–80 μmol of 1 ^{14}C -palmitate per mg mitochondrial protein were used. 100 mM HEPES pH 7.2 was used if not otherwise stated. This buffer was chosen because it allowed the various substrates to be oxidized without significant changes in oxidation of any of the substrates (see Results). Incubation temperature was 35°C, incubation time 2 min, and amount of mitochondrial protein usually 1.1 $\mu\text{g}/\text{ml}$ of



The oxidation of palmitoyl-CoA as a function of incubation time was as stated in Legend to Fig. 1. State 4 conditions (no additions). O—O State 3 conditions (ADP and phosphatase).

oxidations of CoA (A), ATP (B), 1^{14}C -palmitate (C) and L-carnitine (D). The oxidation of palmitate was only moderately stimulated by exogenous CoA, and calculation of an K_m was not possible. It is certainly less than $5 \mu\text{M}$ and thus is in accordance with K_m for palmitoyl-CoA synthetase (Blom *et al.* 1976). The palmitate oxidation was not solely dependent on exogenous ATP (Fig. 3 B). Maximal palmitate oxidation was at palmitate concentrations about 80–100 nmol/mg mitochondrial protein (Fig. 3 C). A saturation concentration of approximately 30 nmol/mg protein (about $40 \mu\text{M}$) is close to the reported K_m for a "purified" palmitoyl-CoA synthetase (Bar Tana *et al.* 1971), very much higher than that reported by Pande *et al.* (1971). The optimal conditions for state oxidation are similar to previously reported optimal conditions for palmitate oxidation (Farstad and Sander 1971). Carnitine-dependence was found with concentrations ≤ 100 nmol palmitate/mg protein, then increasing carnitine independence occurred (Fig.

Fig. 4). Initial rate measurements at concentrations from 0 to 20 nmol/mg protein of all substrates used kinetics for palmitoyl-CoA and palmitoyl-carnitine similar to Michaelis-Menten kinetics, although slightly S-shaped curves were found at very low concentrations. Apparent K_m values calculated from these experiments were about the same as calculated from state saturation curves.

Effects of the incubation conditions on the oxidation of 1^{14}C -palmitoyl-CoA and 1^{14}C -palmitoyl-L-carnitine

Fig. 4 show that with the incubation conditions stated, the formation of acetyl-groups from ^{14}C -palmitoyl-CoA was linear with time up to about 3 min both under state 4 and state 3 conditions. Extending the incubation time over about 4 min also resulted in partial loss of carnitine dependence.

Similar results were found when palmitoyl-L-carnitine was the substrate (not shown).

From data presented in Fig. 1–3 it seems justified to assume that substrate concentrations up to about 50 nmol/mg protein of palmitoyl-CoA and palmitoyl-L-carnitine, and up to about 80 nmol/mg protein of palmitate did not interfere with mitochondrial ability to oxidize palmitoyl-esters or palmitate under state 3 conditions, during the initial 3 min of incubation.

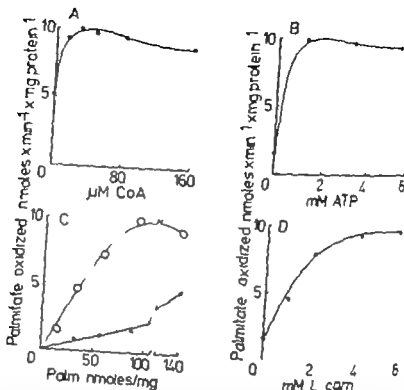


Fig. 3 The formation of ^{14}C -acetyl-groups from ^{14}C -palmitate as a function of the concentration of CoA (A) ATP (B), ^{14}C -palmitate (C) and L-carnitine (D). If not otherwise stated in the text concentration of CoA was 50 μM of ATP 2.5 mM of ^{14}C -palmitate 80 nmoles/mg protein as in Fig. 1. In C ●—● represents absence of L-carnitine. Conditions otherwise as in Fig. 1.

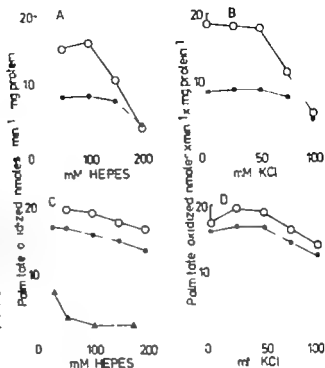
apparent K_m for palmitoyl-L-carnitine was about 10 nmoles/mg protein. Both in presence of phosphate acceptor (state 3) and an uncoupler (CCCP), saturation conditions were somewhat higher and the maximal oxidation rate was increased. In the following experiments 40–50 nmol palmitoyl-L-carnitine per mg protein were used.

Fig. 2 shows the L-carnitine dependent formation of acetyl-groups as a function of concentration of ^{14}C palmitoyl-CoA. At a concentration of L-carnitine of 1 mM, a saturation concentration, or an apparent K_m of 20 nmol palmitoyl-CoA/mg protein was found. At a concentration of palmitoyl-CoA of 50 nmoles/mg protein the apparent K_m for L-carnitine was about 0.1 mM (not shown). As the kinetic for the first enzyme in the Norum 1967 1967 a, 1967 b), a calculation of K_m values is, however, of limited value.

The concentrations of palmitoyl-CoA used (about 50 nmol/mg protein) do not seem to interfere with the formation of acid-soluble products from ^{14}C palmitoyl-CoA under the 3 conditions, although several other mitochondrial functions are known to be affected (Shrago *et al* 1974 Davis and Lumeng 1974 Shug *et al* 1971). Fig. 2 also shows that oxidation of palmitoyl-CoA was dependent on exogenous L-carnitine up to about 60 nmol/mg protein. When palmitoyl-CoA was increased further the β -oxidation became increasingly independent of L-carnitine probably due to increased mitochondrial permeability.

Fig. 3 shows the formation of acetyl-groups from ^{14}C -palmitic acid as a function of

5 Oxidation of palmitoyl-L-carnitine as a function of the concentration of HEPES (A) and KCl presence of 50 mM HEPES and the oxidation of palmitoyl-CoA as a function of the concentration of HEPES (C) and KCl presence of 50 mM HEPES. Conditions otherwise were as in Legend to Fig. 1 and 2. ●—● State 4 conditions (mitochondria). ○—○ State 3 (beams) (ADP and phosphate). ▲—▲ State 4 in the absence of carnitine.



the substrate. When KCl was replaced by 200 mM sucrose in the presence of 50 mM HEPES oxidation rates were further decreased. Fig. 5 furthermore shows that with the substrate concentration used the palmitoyl-CoA oxidation was dependent on exogenous L-carnitine even at 50 mM HEPES. Addition of swelling agent (10 mM phosphate) to the standard incubation mixture increased the oxidation of palmitoyl-esters. The results indicate that the oxidation of palmitoyl-esters was inhibited in hypertonic media, probably due to dehydration of the matrix, in agreement with recently published effect of toxicity on oxidation of palmitoyl-L-carnitine (Osmundsen and Bremer 1976, Christiansen 1977, Nicholls *et al.* 1972).

Activity of palmitoyl-CoA hydrolase from mitochondrial matrix

Recently we have shown (Berge and Farstad 1978) that liver mitochondria contain palmitoyl-CoA hydrolase localized to the inner membrane/matrix. Digitonin fractionation of mitochondria removed almost completely microsomal and lysosomal contamination as the specific activities of microsomal marker and lysosomal marker were about 1-1.5 per cent of the activity in whole homogenates. The specific activity of palmitoyl-CoA hydrolase in matrix was 160 per cent of that in intact mitochondria, and about 110 per cent of that in whole homogenate (Table II). The high hydrolase activity in the matrix suggests a possible role in the intra-mitochondrial metabolism of long-chain acyl-CoA.

TABLE 1 The effect of malate succinate and oxaloacetate on the oxidation of palmitoyl-L-carnitine palmitoyl-CoA under state 4, state 3 and uncoupled conditions. Concentration of ^3H -palmitoyl-L-carnitine was 45 nmole/mg protein, of ^3H -palmitoyl-CoA 50 nmole/mg protein (conditions otherwise as stated in Fig. 1). 2.2 mg mitochondrial protein were used. Concentration Krebs cycle intermediates were 5 mM ADP 1 mM phosphate 1 mM and CCCP 4 μM

Substrate	Nmoles radioactive product/mg protein/min		
	State 4	State 3 (+ADP/P)	Uncoupled
Palmitoyl-L-carnitine	7.8	11.7	10.2
malate	7.1	17.4	15.4
succinate	2.3	7.4	9.8
oxaloacetate	14.0	18.0	15.7
Palmitoyl-CoA	8.1	11.0	7.4
+ malate	10.8	15.2	10.0
+ succinate	5.1	8.8	8.1
+ oxaloacetate	16.2	20.0	11.5

Fig. 1 shows that the presence of CCCP stimulated palmitoyl-L-carnitine oxidation. Other experiments showed that uncoupling stimulated state 4 oxidation of palmitoyl-L-carnitine in freshly prepared mitochondria ($R/C_{ADP} = 5$) whereas an uncoupler had no obvious effect with stored ($R/C_{ADP} = 3$) mitochondria. The effect of CCCP with palmitoyl-CoA was more variable, CCCP rather inhibited palmitoyl-CoA oxidation with some mitochondria.

Swelling seriously interferes with several mitochondrial functions, although little loss of intramitochondrial nucleotides occurs during the first 2-3 min (Bremer and Wojcik 1972). Weighing of the mitochondrial pellets indicated that under the present incubation conditions, the mitochondrial volume increased 15-20 per cent during the first 7 min. The effect of swelling probably are of little importance, although Fig. 5 indicates that hypotonicity stimulated palmitoyl-CoA oxidation in our system.

The oxidation of palmitoyl L-carnitine was stimulated by oxaloacetate and inhibited by succinate both under state 3 and 4 conditions. Malate stimulated only under state 3 conditions (Table 1). Addition of an uncoupler (CCCP) stimulated palmitoyl L-carnitine oxidation both in presence of malate and succinate, but not in the presence of oxaloacetate. Similar effects were observed with palmitoyl-CoA as the substrate both under state 3 and state 4. With this substrate, however, addition of CCCP partly reversed the effect of succinate and oxaloacetate.

Effect of tonicity on the oxidation of palmitoyl L-carnitine and palmitoyl-CoA

Fig. 5 shows that both palmitoyl L-carnitine and palmitoyl CoA were oxidized at a higher rate in a hypotonic medium than in an isotonic medium. Notably state 3 oxidation of palmitoyl L-carnitine was very high in hypotonic buffers. It is also seen that increasing tonicity had a more pronounced effect with palmitoyl L-carnitine than with palmitoyl-CoA.

- ▣ Observed rates for β -oxidation of palmitate or palmitoyl-esters in relation to formation of palmitoyl-CoA and hydrolysis of palmitoyl-CoA by rat liver mitochondria. Data are extracted from Figures and Tables of this report and compared to results extracted from recent publications.

	Nmoles substrate/ min, mg protein	
rate of palmitate	11	
rate of palmitoyl-CoA	11	
rate of palmitoyl-L-carnitine	9	
rate of palmitoyl-CoA	30	
rate of palmitoyl-CoA	9	12 ^a
palmitoyl-CoA synthetase	70 ^b	23 ^c
rate palmitoyltransferase	16 ^d	
rate of palmitoyl-L-carnitine	3.5 ^e	
rate of palmitoyl-CoA	3.5 ^e	4 ^d

^a von Beyer and Farstad (1978)

^b von Arn (1971) 35^a

^c von Pande (1971) 28^a

^d von McMillin Wood *et al.* (1977), calculated assuming 46 nmoles 11 consumed per nmole palmitate

2.7) Whereas both palmitate and palmitoyl-L-carnitine was significantly oxidized at a concentrations of 12 mg/ml (4 nmol/mg protein), the oxidation of palmitoyl-CoA was only zero at about 6 mg BSA/ml (3 nmol/mg protein). In the intact cell, the cytosolic concentration of 50–60 mg protein/ml indicates that extramitochondrial competition for palmitoyl-CoA formed most likely is of great importance for the availability of substrate for oxidation.

Table III summarizes data derived from Fig. 6, Fig. 7 and Table I compared to reported values for oxidation of palmitoyl-esters and for mitochondrial activities of palmitoyl-CoA synthetase and carnitine palmitoyltransferase. Table III shows that the activities of enzymes involved in palmitate activation and translocation are very much higher than reported values for palmitate oxidation in rat liver (Pande 1971; McMillin Wood *et al.* 1977). The maximal capacity for β -oxidation in the present investigation was, however, very much higher than the values obtained by measuring O_2 -consumption (Pande 1971).

Also the activity of palmitoyl-CoA hydrolase must be brought into consideration as the rate in the mitochondrial matrix was as high as 1 nmol/mg matrix protein, and possibly may compete with the acyl-CoA dehydrogenase for intramitochondrial palmitoyl-CoA.

Discussion

The present results indicate higher initial oxidation rates of palmitate, palmitoyl-CoA and palmitoyl-L-carnitine *in vivo* under maximally stimulated conditions than previously reported, up to 20 nmol/min/mg protein. This is about 3 times previously reported values (Pande 1971; McMillin Wood *et al.* 1977), when corrected for differences in incubation temperature. The present results were obtained in hypotonic buffer and with substrate concentrations that interfere with several mitochondrial functions (Shrago *et al.* 1974; Davis and

TABLE II The activity of palmitoyl-CoA hydrolase in homogenate of mitochondria and isolated matrix in relation to a microsomal marker (NADPH cytochrome C reductase) and a lysosomal enzyme (acid phosphatase). Values in parentheses represent the specific activity as per cent of the specific activity in whole liver homogenate (For details see Berge and Farstad 1978)

	Specific activity (nmoles/min/mg protein)		
	NADPH cytochrome c reductase	Acid phosphatase	Palmitoyl-CoA hydrolase
Mitochondria	870	3.2	5.0
Isolated matrix	174 (1.5)	0.4 (1)	9.0 (110)

A possible rate limiting step in the oxidation of palmitate by rat liver mitochondria: Availability of palmitoyl CoA

From the data presented in Table I the maximally stimulated palmitoyl-ester oxidase was very high, up to about 70 nmol/min/mg protein in some expts. A possible rate limiting factor in the oxidation of palmitate in the intact cell is the availability of palmitoyl-CoA. Fig. 6 shows the formation of ^1C -acetyl-groups and the formation of palmitoyl-CoA from ^1C palmitate, CoA and ATP. During the first 2 min the formation of palmitoyl-CoA was higher than the oxidation of the palmitoyl-CoA formed. The initial rate of palmitoyl-CoA formation was about 25–30 nmoles per mg protein per min, but levelled off after about 1 min. Palmitate oxidation was low the first minute, then increased to about 10 nmol/mg protein. This was probably due to initial binding of palmitoyl-CoA to mitochondrial protein and insufficient palmitoyl-CoA was available for the carnitine palmitoyltransferase.

Addition of BSA resulted in a very strong inhibition of the oxidation of all substrates

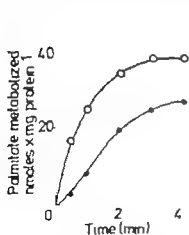


Fig. 6

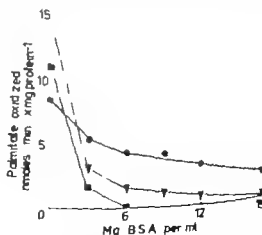


Fig. 7

Fig. 6. The formation of palmitoyl-CoA (O—O) and acetyl-groups (●—●) from ^1C -palmitate as a function of time by rat liver mitochondria (1.2 mg protein/ml). ^1C -palmitoyl-CoA was extracted according to Bar Tana *et al.* (1971). Incubation conditions were as described in Legend to Fig. 3

Fig. 7. The effect of BSA on the oxidation of palmitoyl-L-carnitine (●—●), palmitoyl-CoA (■—■) and palmitate (▼—▼) in state 4. Conditions were as stated in Legend to Fig. 1. 3. The concentrations of the three substrates were 60, 60 and 95 $\mu\text{mol/l}$, respectively

Table II Observed values for β -oxidation of palmitate or palmitoyl-esters in relation to formation of palmitoyl-CoA and hydrolysis of palmitoyl-CoA by rat liver mitochondria. Data are extracted from Figures and Tables of this report and compared to results extracted from recent publications.

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rate of palmitoyl-CoA	30	
rate of palmitoyl-CoA	9	17 ^a
rate of CoA in substrate	70 ^b	23 ^c
rate palmitoyl transference	16 ^d	
rate of palmitoyl-L-carnitine	3.5 ^e	
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^a van Berge and Farnold (1978)
^b van Aals (1977) 35°
^c van Pande (1971) 23°
^d van McMillan Wood *et al.* (1977), calculated assuming 46 acetates O consumed per nmole palmitate
^e *ibid.*
^f *ibid.*

Fig. 7 Whereas both palmitate and palmitoyl-L-carnitine was significantly oxidized at a concentration of 1 mg/ml (4 nmol/mg protein), the oxidation of palmitoyl-CoA was only zero at about 6 mg BSA/ml (8 nmol/mg protein). In the intact cell, the cytosolic concentration of 50-60 mg protein/ml indicates that extramitochondrial competition for palmitoyl-CoA formed most likely is of great importance for the availability of substrate for oxidation.

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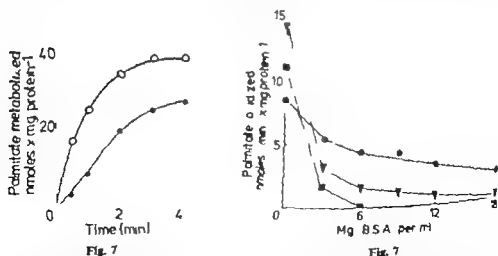


Fig. 6. The formation of palmitoyl-CoA (O—O) and acetyl-groups (●—●) from ^3H -palmitate as a function of time by rat liver mitochondria (11.2 mg protein/ml). ^3H -palmitoyl-CoA was extracted according to Bar-Tana *et al.* (1971). Incubation conditions were as described in Legend to Fig. 1.

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Lumeng 1974) The mitochondria seemed, however functionally intact with respect to β -oxidation. This assumption is based on the following

- 1 β -Oxidation increased under state 3 conditions (ADP-stimulated).
- 2 β -Oxidation of palmitoyl-CoA was higher at concentrations independent of L-carnitine than at more physiological concentrations, indicating that hypotonicity increased β -oxidation
- 3 Uncoupling stimulated the palmitoyl-ester oxidation in fresh mitochondria, but not in aged mitochondria.

The presence of L-carnitine has a beneficial effect on the palmitoyl-CoA inhibition of adenine nucleotide translocases (Shug *et al* 1971) and palmitoyl-CoA up to 20 and 25 μ M protein has no effect on substrate level phosphorylation, phosphate transport, and ATPases when incubation time is less than 5 min (Ho and Pande 1974). Also calcium-induced swelling has little effect on mitochondrial coupling (Hunter *et al* 1976). Rat liver mitochondria oxidize palmitoyl-carnitine and palmitoyl-CoA optimally at 10–20 μ M of protein (McMillin Wood *et al* 1977) assayed by the oxygen consumption. The higher activities obtained by us are probably due to a short incubation, as the effects of palmitoyl esters are time dependent (Ho and Pande 1974). Swelling of the mitochondria increased β -oxidation in the present investigation, probably because of carnitine-independent transport of palmitoyl-CoA through the more permeable inner membrane (Fig. 2).

The effects of Krebs cycle intermediates are in agreement with well-known observations that changes in the NADH/NAD ratio and competition at the flavoprotein step in the respiratory chain are major regulatory steps in fatty acid oxidation and ketogenesis (Berg and Wojtczak 1972, Lumeng *et al* 1976, Lopez-Cardozo and van den Bergh 1972, Vampour *et al* 1972). The finding that succinate was less inhibitory with palmitate or palmitoyl-CoA as substrates than with palmitoyl-L-carnitine as substrate may however indicate that the respiratory chain will not be the rate limiting step under physiological conditions.

The present results indicate that the β -oxidation cycle is less sensitive to changes in mitochondrial integrity than the Krebs cycle linked respiration (Pande 1971, McMillin Wood *et al* 1977), and that state 3 respiration can be obtained at very high substrate concentrations in agreement with Rafael and Wraebel (1976).

From the present results it seems warranted to conclude that all steps involved in fatty acid activation, translocation over the inner mitochondrial membrane, β -oxidation and respiratory chain transport are sufficiently active to meet all fatty acid oxidation taking place in the intact cell. In the cytosolic space of rat liver the calculated acyl-CoA probably never exceeds 1 nmol/mg protein according to available data (Nordlie *et al* 1967). This seems likely that the availability of long-chain acyl-CoA will be the dominating regulatory factor of fatty acid oxidation in the intact rat liver cell. On the other hand the capacity of β -oxidation in the present study is in accordance with the β -oxidation in intact hepatocytes calculated to be up to 8–10 nmol/min/mg mitochondrial protein under stimulated conditions (Christiansen 1977).

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Effects of dopaminergic agonists and antagonists on isolated cerebral blood vessels

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Abstract

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The vasomotor response of dopamine and dopaminergic agonists was studied *in vitro* on middle cerebral arteries from cat and pial arteries from baboon. The action of various inhibitors was tested in order to define the receptors involved. A contractile response could be obtained by epinephrine, apomorphine and levamisole in the mentioned order of potency. The effect was blocked by α -receptor as well as serotonin receptor antagonists. The mode of inhibition suggested that serotonin receptors rather than α -adrenoceptors mediated the dopamine-induced contraction. A dose-dependent dilatation could be evoked by the dopaminergic agonists on actively contracted pial arteries. The relative potency was epinephrine > apomorphine > levamisole. The order of potency for the agonists, together with blocking experiments (including parallel shift in the log dose-response curve induced by halocapazine), indicated that the vasodilatation is mediated by specific dopamine receptors.

Ever since it became clear on the basis of chemical determinations that dopamine has a regional distribution within the brain (Bertler and Rosengren 1959; Bertler 1961) and that it was established that the catecholamine was localized within the neurons in the central nervous system (Carlsson, Falck and Hillarp 1962), numerous studies have been carried out to describe the organization and function of the central dopaminergic systems. As the histochemical techniques have been improved in sensitivity and specificity many dopamine systems, which are previously undetected, have been disclosed recently. The central dopamine projection systems which have been characterized at the present time (see Lindvall and Björklund 1977 for review) are the mesostriatal, the mesocortical, the periventricular, the mesencephalic, and the tuberohypophyseal systems as well as the periglomerular dopamine neurons.

These widespread dopaminergic systems are closely associated with microvessels within the brain. Although dopaminergic neurons have not, as yet, been shown to terminate directly on cerebral blood vessels, this type of arrangement has been proposed to occur in regions such

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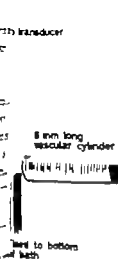


Fig. 1

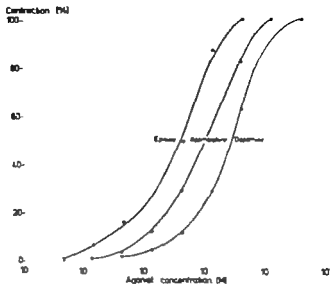


Fig. 2

Fig. 1. Arrangement for suspension of vascular cylinder between two L-shaped metal prongs for recording of vascular contractile activity.

Fig. 2. Contractions in response of cat's middle cerebral artery in response to epinephrine, apomorphine, and dopamine to show relative agonist potency in a representative experiment.

Concentrations below are given as the salt and expressed as the final molar concentration in the buffer solution of the organ bath.

Analysis of data. Concentration-response curves were obtained by cumulative application of the drugs to the organ bath. The irreversible antagonist, phenoxybenzamine, was present for 30 min in the organ bath followed by several washouts before the agonists were tested again. The reversible inhibitory drugs phentolamine, pargoline, cyproheptadine, methysergide, haloperidol, flusocaprine and propranolol were added 10 min before and were present during the tests. In cases in which the antagonism of the response involved a decrease in the maximum and the slope of the actual log dose-response curves, the E_{50} of the curve before addition of the antagonist was set as 100 and the subsequent curves obtained after blockade were related to this value. When antagonism only involved parallel shift in the actual log dose-response curves, the E_{50} of each curve from the experiment was set as 100. The response was also characterized with regard to the concentration of the drug resulting in half-maximum response (ED_{50}). The apparent dissociation constant for the receptor-agonist complex (K_D) was calculated according to the equation $[A]^2/[TA] - 1 = [D]K_D$, where $[A]^2/[A]$ (dose ratio) is the ratio of concentrations of agonist giving an equal response in the presence and in the absence of a given concentration of the antagonist, $[D]$ (Furchgott 1972). Calculations of differences between mean values were carried out with a Hewlett-Packard desk computer.

Results

Contractile response

The preparation used (feline middle cerebral artery and small human pial arteries) showed no spontaneous contractions. Under resting conditions, i.e. when the vessels had reached a steady level of tension (approximately 300 dyn), epinephrine, apomorphine and dopamine all produced a contractile response. The effect fairly rapidly reached a plateau, which was main-

as the caudate-putamen (Hartman, Zide and Udenfriend 1972). It has recently been suggested that in addition to any direct vasomotor influence dopamine may exert upon cerebral vessels, the manipulation of intracerebral dopaminergic systems may alter cerebral blood perfusion indirectly namely by changing the metabolic needs of the brain (McCulloch and Harper 1977).

Specific dopamine receptors have been suggested to occur in renal, mesenteric and coronary vessels but not in the femoral and carotid vasculature (see review by Goldberg 1977). Recent studies have demonstrated that dopamine is able to both contract and dilate cerebral blood vessels *in vitro* (Toda *et al* 1975, Toda 1976, Edvinsson *et al* 1977). This has been confirmed under *in vitro* conditions using the microapplication technique where dopamine was found to constrict pial arterioles on the surface of the cerebral cortex of cats and apomorphine, a dopamine receptor antagonist, dilated these vessels (Edvinsson *et al* 1977). Little is, however, known about the detailed receptor mechanisms mediating this dopamine action on the cerebrovascular bed. In order to define the nature of the receptors involved, dopaminergic agonists and antagonists have been tested on segments of cerebral vessels *in vivo*.

Material and methods

Animals. Specimens of the middle cerebral artery were obtained from 15 adult cats of either sex, eight between 1.8 and 4.5 kg. All animals were killed by bleeding under sodium pentobarbital anaesthesia, the head was removed, and the pial vessels were immediately dissected out and placed in aerated Krebs-Ringer buffer solution (for composition, see below). Part of the material was immediately used in the experiments, the rest was used after storage in the buffer solution in a refrigerator (+4°C) for up to 24 h.

Denervation. Sympathectomy was carried out under sodium pentobarbital anaesthesia (30 mg/kg i.v.) in 5 of the animals by unilateral or bilateral excision of the superior cervical ganglia at least 1 week prior to the experimentation. These preliminary tests did not demonstrate any significant difference between the two types of vessels with regard to their response to dopaminergic agonists. Therefore the majority of experiments were carried out on innervated middle cerebral arteries.

Human material. During lobe resection in conjunction with neurosurgical tumor operations, small segments of human pial arteries on the surface of the temporal and parietal lobes were dissected free, mounted in ice-cold Krebs-Ringer buffer solution and immediately transported (15 min) to the laboratory and mounted as above for registration of vasomotor activity.

Recording of vasomotor effects. Small segments of the middle cerebral artery of cats, about 300–400 μ m wide and 5 mm long (Fig. 1), and small surface pial arterioles of similar size from humans were suspended in an organ bath containing 50 ml of bicarbonate-buffered Krebs-Ringer solution. The solution was aerated continuously with 95% O₂–5% CO₂ mixture and maintained at 37°C. The composition of the Krebs-Ringer solution (millimoles per liter) was: NaCl 118, KCl 4.5, CaCl₂ 2H₂O 2.5, MgSO₄ × 7H₂O 1.0, KH₂PO₄ 1.0, NaHCO₃ 25 and glucose 6.0. Washout of the chamber was made by renewing its total content. The segments were suspended between two L-formed metal prongs attached to Endevco Model 8122 isometric force-displacement transducers for recording of circular contractile activity on a Grass Model 7B polygraph. The basal tension on each vessel segment (2 segments were suspended in the same bath) was adjusted to approximately 400 dyn after which it was allowed to relax and accommodate for 2 h prior to the addition of any drug (Edvinsson, Nielsen and Öрман 1974, Edvinsson and Öрман 1974).

Drugs. The following compounds were used: Apomorphine hydrochloride (ACO, Sweden), L-arteriole hydrochloride (Sigma), epinine hydrochloride (Regis), dopamine hydrochloride (Nutritional Biochem. Corp., USA), 5-hydroxytryptamine creatinine salt (Sigma), DL-isoproterenol hydrochloride (Sigma), bulbo-capaine (Smith, Kline and French), cyproheptadine (Merck, Sharp and Dohme), haloperidol (AB Leo, Sweden), methysergide hydrogenmaleate (Sandoz), phenoxymethylamine hydrochloride (ACO, Sweden), phenolamine methanesulphonate (Hänsle, Ciba, Geigy), pinoxidol (AB Leo, Sweden), papaverine hydrochloride (Rhône-Poulenc), propranolol hydrochloride (Draco, Sweden). All drugs were dissolved in 0.9% saline (bulbo-capaine and pinoxidol after adding a few μ l of 1M HCl).

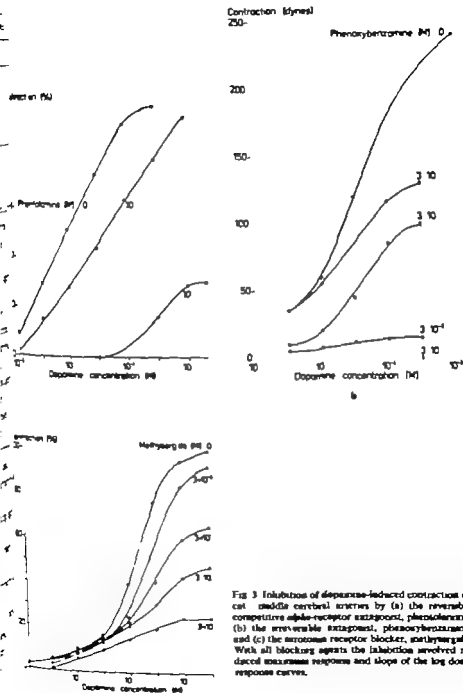


Fig 3 Inhibition of dopamine-induced contraction of cat middle cerebral arteries by (a) the reversible competitive α_1 -receptor antagonist, phentolamine (b) the irreversible antagonist, phenoxybenzamine, and (c) the serotonin receptor blocker, methylergometrine. With all blocking agents the inhibition involved reduced maximum response and slope of the log dose-response curves.

TABLE 1 Contractile response of feline isolated middle cerebral arteries to dopaminergic compounds in comparison with norepinephrine and 5-hydroxytryptamine. Mean values \pm S.E.

Agonist	Number of tests	ED ₅₀ values (M)	E _{max} values (%)
Dopamine	14	$(3.80 \pm 1.41) \cdot 10^{-6}$	327 ± 55
Epinephrine	4	$(6.17 \pm 1.29) \times 10^{-6}$	227 ± 56
Apomorphine	7	$(1.00 \pm 1.29) \times 10^{-6}$	83 ± 19
Norepinephrine	10	$(2.17 \pm 0.55) \cdot 10^{-6}$	233 ± 46
5-Hydroxytryptamine	15	$(3.45 \pm 1.01) \cdot 10^{-6}$	304 ± 46

tained long enough to allow for cumulative application of increasing doses. After rising, the arteries returned to their initial level of tension.

In some instances the experiments were also performed on sympathectomized arteries from cat (1 week postoperatively). No difference was seen in the response between vessels from operated and non-operated animals. The same pattern of response under the various conditions given below was noted in preparations from both feline and human vessels.

Relative potency. In order to evaluate the relative potency of the agents mentioned above with regard to their contractile effects, full dose-response curves were constructed, and the mean concentrations required to obtain half-maximum contraction (ED₅₀) were compared (Fig. 2). The relative potency was epinephrine > apomorphine > dopamine, as summarized in Table 1. However, dopamine produced the strongest contractile response, which was of the same order of magnitude as that produced by 5-hydroxytryptamine (Table 1).

Competitive antagonism. If the contractile effect of the dopaminergic agents is mediated by α -adrenergic receptors, reversible competitive antagonists, such as phentolamine and piperoxan, should decrease the sensitivity of the test system to the agonists without decreasing the maximum or the slope of the log dose-response curve (Furchgott 1972). This was tested by using dopamine as the agonist. It was found that both phentolamine (Fig. 3a) and piperoxan in increasing concentrations caused a decrease in the slope and the maximum of the dose-response curves, suggesting that the inhibitory effect on the dopamine-induced contraction was not mediated specifically by α -adrenergic receptors.

The β -haloalkylamine, phenoxybenzamine, is a competitive blocking agent which, for practical purpose, can be considered as irreversible through its ability to inactivate the receptor sites (Furchgott 1972). In the present study, increasing doses of phenoxybenzamine caused a dose-dependent reduction in the slope and in the maximum contractile effect (Fig. 3b). A dose of $3-5 \times 10^{-6}$ M phenoxybenzamine was required to completely abolish the dopamine induced contraction (Fig. 3b).

The two serotonin receptor antagonists, methysergide (Fig. 3c) and cyproheptadine, also inhibited the dopamine induced contraction by reducing the maximum and the slope of the dose-response curves in the same way as previously found with 5-hydroxytryptamine as agonist in cerebral blood vessels (Edvinsson, Hardebo and Owman 1978).

Dilatory response

In studies on the dilatory effect of dopaminergic agonists, the pial arteries were exposed to $3-5 \times 10^{-6}$ M phenoxybenzamine for 30 min to block their contractile effects. Furthermore,

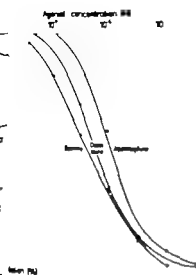


Fig. 5

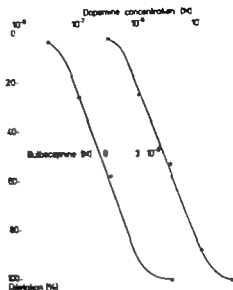


Fig. 6

Fig. 5. Relative dilatory effect of cat's middle cerebral artery obtained by epinephrine, dopamine, and apomorphine (after blocking contraction by exposure to $3-5 \times 10^{-6}$ M phenylephrine for 30 min and after the vessel is active tone by prostaglandin $F_{2\alpha}$).

Fig. 6. Parallel shift of dilatory log dose-response curve for dopamine in the presence of halobucaprine. Administered as in Fig. 5.

of increasing concentrations of halobucaprine (Fig. 6). The apparent K_{50} value was found to be $(1.65 \pm 0.51) \times 10^{-6}$ M from 10 tests. However in high concentrations (above 10^{-5} M) this antagonist caused a vascular dilatation of its own.

The reversible β -adrenoceptor blocking agent, propranolol, known to cause a marked parallel shift of the dose-response curve to isoproterenol (Edvinsson and Owman 1974) was without effect on the dopamine-induced relaxation.

Discussion

Dopamine has complex vascular effects and may both contract and relax blood vessels (Cohen and Berkowitz 1975). Attempts to elucidate the mechanism(s) for the effects produced by dopamine have included reports of receptors specific to dopamine in ganglia (Kebabian and Greengard 1971), brain (Kebabian, Petzold and Greengard 1972) and in arteries within mesenteric, renal (McNay and Goldberg 1966, Yeh, McNay and Goldberg 1969, Higgins *et al.* 1973) and coronary (Schuelke *et al.* 1971) vascular beds. Apomorphine has been used as a tool to confirm the presence of dopamine receptors since it has been shown to possess structural (Goldberg, Sonnevillie and McNay 1968), biochemical and functional (Ardán *et al.* 1967) effects similar to dopamine.

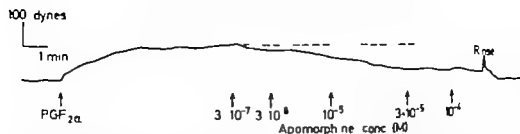


Fig. 4 Representative experiments showing tonic contraction induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the dilatory effect of apomorphine in increasing concentrations. The dashed line indicates the steady level of contraction in the presence of $PGF_{2\alpha}$ alone.

in order to reveal a clear-cut dilatory response the arteries were given an active tonic contraction by adding $2.5 \cdot 10^{-6}$ M prostaglandin $F_{2\alpha}$. This caused in the present series of experiments a strong contraction amounting 560 ± 92 (mean \pm S.E.) dyn, which remained at a steady level for more than 20 min, i.e. long enough to allow for cumulative application of various agents (Fig. 4).

Relative potency. The ability to produce a dilatation (relaxation) of the feline middle cerebral artery and human pial arteries was tested under the conditions described above (Fig. 4). The relative potency (comparison of mean ED_{50} values) was found to be epinephrine > dopamine > apomorphine (Table II). The effects were dose-dependent and amounted 30–90 dyn (Fig. 5). For comparison, isoproterenol dilated the preparation by about 120 dyn, and the ED_{50} values were lower than for the dopaminergic agonists. Relaxations by dopamine and dopaminergic agents were obtained in about 50% of the experiments. In others either no effect or a contraction was obtained. If a further dose of phenoxybenzamine was applied the contraction could be abolished and sometimes a dilatation could be demonstrated.

Competitive antagonism. Experiments were undertaken with several putative dopamine receptor antagonists to reveal the characteristics of the receptor involved in this dopamine-induced dilatation. Low concentrations of haloperidol (less than 10^{-6} M) did not shift the dopamine-induced dilatation towards higher doses. Greater concentrations of haloperidol (i.e. concentrations at which the drug acts as a dopamine antagonist at other sites) dilated the preparation to the same degree as dopamine and in the concentrations supposed to be antagonistic. Pimozide, another dopamine receptor antagonist, could not be dissolved in the buffer solution used.

Bulbocapnine, which has recently been suggested to act as a specific dopamine receptor antagonist, was able to alter the dopamine curve towards higher doses, i.e. the sensitivity of

TABLE II Dilatation of the middle cerebral artery after blockade with $3 \cdot 10^{-6}$ M phenoxybenzamine and after giving the vessels an active tonic contraction amounting 560 ± 92 dynes by prostaglandin $F_{2\alpha}$ ($2.5 \cdot 10^{-6}$). Results are expressed as mean values \pm S.E.

Agonist	Number of tests	ED_{50} values (M)	ED_{50} values (dyn)
Dopamine	13	$(8.45 \pm 1.36) \cdot 10^{-7}$	92 ± 20
Epinephrine	6	$(3.83 \pm 1.21) \cdot 10^{-7}$	31 ± 4
Apomorphine	5	$(9.42 \pm 3.21) \cdot 10^{-6}$	90 ± 19
Isoproterenol	10	$(1.26 \pm 0.63) \cdot 10^{-6}$	116 ± 20

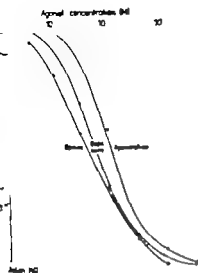


Fig. 5

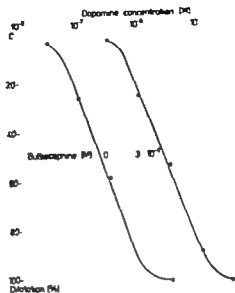


Fig. 6

5. Relative dilatory effect of cat's middle cerebral artery obtained by epinephrine, dopamine, and apomorphine (after blocking contraction by exposure to $3-5 \times 10^{-6}$ M phenoxybenzamine for 30 min and after ring the vessel as active tone by prostaglandin $F_{2\alpha}$).

6. Parallel shift of dilatory log dose-response curve for dopamine in the presence of sulbocapsine, shown as in Fig. 5.

response to dopamine (and in a few experiments apomorphine) was reduced by administration of increasing concentrations of sulbocapsine (Fig. 6). The apparent K_d value was found to be $(1.65 \pm 0.51) \times 10^{-7}$ M from 10 tests. However in high concentrations (above 10^{-4} M) this antagonist caused a vascular dilatation of its own.

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Dopamine has complex vascular effects and may both contract and relax blood vessels (Cohen and Berkowitz 1975). Attempts to elucidate the mechanism(s) for the effects produced by dopamine have included reports of receptors specific to dopamine in ganglia (Kebabian and Greengard 1971), brain (Kebabian, Petzold and Greengard 1972) and in arteries within mesenteric, renal (McNay and Goldberg 1966, Yeh, McNay and Goldberg 1969, Higgins *et al.* 1973) and coronary (Schuelke *et al.* 1971) vascular beds. Apomorphine has been used as a tool to confirm the presence of dopamine receptors since it has been shown to possess structural (Goldberg, Sonnevile and McNay 1968), biochemical and functional (Anders *et al.* 1967) effects similar to dopamine.

Contractile response

Dopamine, apomorphine and epinine all constricted the feline middle cerebral artery in a dose-dependent way at relatively high doses. The contractile effect of dopamine could be attenuated by relatively high concentrations of the adrenergic blocking agents phentolamine, piperoxan and phenoxybenzamine. Dopamine induced contractions have been demonstrated in peripheral arteries and veins, both in isolated vessels and in intact vascular beds. These responses are due to the activation of α -adrenergic receptors (cf. Goldberg *et al.* 1975). In rabbit aorta the contraction produced both by apomorphine and dopamine could be blocked by dibenamine, suggesting that both agonists contract this vascular smooth muscle by a similar α -adrenergic receptor stimulation (Cohen and Berlowitz 1971). Previous work on cranial vessels has shown a constrictor response following administration of dopamine to the isolated middle cerebral and basilar arteries (Toda 1976), and a vasoconstriction has also been revealed under *in vivo* conditions (White, Hagen and Robertson 1977; Edvinsson *et al.* 1977). This constrictor response was abolished by the α -receptor blocking agents. During infusion of small doses of dopamine (< 1 mg/kg/min h) to dogs, the cerebral blood flow is reduced, which has been ascribed to an effect on α -adrenergic receptors (von Essen 1974). In the present study it was found that the competitive reversible α -receptor antagonist, phentolamine and piperoxan, did not cause a parallel shift of the dose-response relation of dopamine but rather reduced the slope and maximum effect, which is not consistent with a clear-cut action of dopamine on α -adrenergic receptors. In other studies, a dose of 3×10^{-6} M of phenoxybenzamine was needed to totally block a norepinephrine induced contraction (Edvinsson and Öwman 1974). This concentration reduced the response to serotonin but did not abolish it (Edvinsson, Hardebo and Öwman 1978). Since a concentration of $3\text{--}5 \times 10^{-6}$ M phenoxybenzamine was required to abolish the dopamine induced contraction, the findings would indicate that the dopamine induced cerebral vasoconstriction is mediated at least in part by other receptors than α -adrenergic. A similar conclusion was reached by Gilbert and Goldberg (1975) in experiments on canine femoral and carotid arteries. They observed that the 5-hydroxytryptamine receptor blocking agent, cyproheptadine inhibited contractions induced by dopamine, tryptamine and 5-hydroxytryptamine in both types of arteries to a similar extent, whereas the norepinephrine induced contractions were unaffected. Protection experiments suggested that the vasoconstriction evoked by dopamine is to a large extent mediated by serotonin receptors. The same mechanism may hold true also for the brain vessels, where cyproheptadine and methysergide were able to inhibit both dopamine- and 5-hydroxytryptamine-induced contractions, without affecting the contractile effect of norepinephrine (Edvinsson *et al.* 1978). The relatively high doses of phenoxybenzamine required to block the dopamine response is consistent with the dose level at which the 5-hydroxytryptamine-induced contraction of placental arteries is blocked.

Dilatory response

In addition to contraction of the placental vessels, relaxation can be observed following phenoxybenzamine blockade. After this treatment neither serotonin nor α -adrenergic

prior agonists were capable of producing a satisfactory contraction, and therefore nifedipine F_{50} was used to induce the active contractile tension in these preparations. The pial arteries dilated (relaxed) upon administration of the dopaminergic compounds in the following order of potency: epinephrine > dopamine > apomorphine. It has been suggested in experiments on aortic strips that the effect of dopamine is mediated by an increase in cyclic AMP levels (Cohen and Berkowitz 1975). In accordance with this, administration of cyclic AMP has been found to produce a dose-dependent relaxation of pial artery preparations (Hardebo and Edvinsson 1979). Apomorphine, in concentration that produced the same degree of relaxation as dopamine, was less effective in altering cyclic AMP levels (Cohen and Berkowitz 1975). This is in accordance with the present study where dopamine was found to be more effective than apomorphine in dilating the pial vessels. When the α_1 -receptors are blocked, dose-related vasodilatations have been shown to occur in the renal and mesenteric vascular beds after intra-arterial injections of dopamine (Goldberg 1972) and there is some evidence that, within the cerebral vascular bed, blood flow may also be increased in similar circumstances (von Eenen 1974).

Dopamine increases cardiac contractility and heart rate (Goldberg 1975); these actions are antagonized by propranolol, indicating that they are due to stimulation of cardiac β -receptors. On the other hand, dilatation of the pial arteries caused by dopaminergic agents is not affected by propranolol in concentrations sufficient to cause a marked shift in the dose-response curve of isoproterenol. Similar findings have been obtained from studies on dilated renal, mesenteric, coronary and femoral arteries. Moreover this vasodilatation was not affected by atropine, antihistaminics, or by pretreatment with reserpine, compound 480, or monoamine oxidase inhibitors (see Goldberg 1972, 1975).

There is reason to assume that the dopaminergic agents act on a specific dopaminergic receptor to produce dilatation in peripheral arteries. In the present study attempts to descriptively characterize the receptor by which dopamine dilates cerebral arteries have been only partially successful, although a number of pieces of circumstantial evidence point to it being a specific dopamine receptor. The dopamine-induced relaxation of renal and mesenteric vessels has been antagonized selectively by haloperidol (Goldberg 1972). In the present study this was not possible to achieve since haloperidol itself relaxed the pial arteries in doses required to inhibit the vasodilatation produced by dopamine. On the other hand however, bupropazine, a peripheral dopamine receptor antagonist, shifted the dose-response curve of epinephrine in isolated pial arteries towards higher doses. Bupropazine has a structural similarity with dopamine and apomorphine and has been shown to antagonize peripheral vascular dopamine effects, probably in a competitive way (Pendleton, Finlay and Sberman 1975, Setler, Pendleton and Finlay 1975). Bupropazine possesses some α_1 -adrenolytic (Van Rossum 1965), but no anticholinergic, antihistaminic, or β -adrenolytic activity. However, bupropazine, in doses above $1 \cdot 10^{-4}$ M exerts a dilatory effect on isolated artery segments. It is not unlikely that this dilatation is the result of a dopamine agonistic property of bupropazine.

Recent investigation have demonstrated that the administration of apomorphine results in increases in cerebral blood flow accompanied by an elevated cerebral oxygen and glucose consumption (McCulloch and Harper 1977). It was suggested that the primary action of

Contractile response

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Dilatory response

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apomorphine was to stimulate cerebral metabolic activity and that the increased cerebral tissue perfusion was secondary to this. The preliminary observations (Edvinsson *et al.* 1977) of apomorphine-induced dilatations of the pial arterioles on the convexity of the brain following perivascular microapplication, together with the *in vitro* experiments, suggest that direct action of apomorphine upon the cerebral vasculature may contribute to the increase in cerebral tissue perfusion noted with this agent (McCulloch, Teasdale and Harper 1977). The observations of dopamine-induced changes in cerebral blood flow by von EsSEN (1974) although subject to methodological criticism, could be considered to be consistent with both the observations *in vitro* and *in vivo* of the present study.

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4.1 definition of the extraction. We consider an experimental situation in which a number n of molecules (ions), n , is injected as a bolus into the artery of an organ. After injection at $t = 0$, the number of injected molecules can be divided according to their history at t , such that

$$n = N_E(t) + N_T(t) + N(t) \quad (1)$$

where $N_E(t)$ is the number of molecules that have been extracted (i.e. passed the capillary membrane at least once) at time t , $N_T(t)$ is the number of molecules that have been transmitted (i.e. passed the capillaries without having been extracted) at time t and $N(t)$ is the number of molecules that have been neither extracted nor transmitted at time t .

There exists a time $t_0 > 0$, such that

$$n = N_E(t) + N_T(t) \quad (2)$$

The extraction is

$$E = N_E(t)/n, \text{ for } t > t_0 \quad (3)$$

4.2 Concentration of test molecules. We assume that the number of injected molecules can be measured in a fixed part of the organ by external detection. The detector signal can be expressed as

$$S(t) = n - N_d(t) \quad (4)$$

and

$$S(t) = N_d(t) - N_a(t) + N_{av}(t) + N_v(t) \quad (5)$$

valid for $t > t'$ where t' is the time when the last molecule in the bolus has entered the detector field. The subscript d denotes the molecules present in the venous blood outside of the detector field and the subscripts a , v , av and v denote the molecules in the arteries, the capillaries, the extravascular space and the veins in the detector field, respectively.

The number of molecules that has traversed the capillary membrane at least once at time t can be expressed in terms of their position as

$$N_E(t) = N_{av}(t) + N_{E,d}(t) + N_{E,v}(t) + N_{E,av}(t) \quad (6)$$

Similarly the molecules that have been transmitted at time t can be expressed in terms of their position as

$$N_T(t) = N_v(t) - N_{E,v}(t) + N_a(t) - N_{E,a}(t) \quad (7)$$

where

$$N_d(t) = N_{E,d}(t) + N_{T,d}(t) \quad (8)$$

At time $t > t_0$ we have that

$$N_d(t) = 0 \quad (9)$$

$$N_a(t) = N_{E,a}(t) \quad (10)$$

4.3 Proposition. According to Sejrsoen (1970), the function

$$F(t) = N_{av}(t) \quad (11)$$

A note on the single injection residue function method to determine capillary permeability

By

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Abstract

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The assumptions inherent to the single injection residue function method to determine capillary permeability have been made explicit. Furthermore, it is shown that agreement between the deduced extraction of the transmitted molecules of the test substances and that of reference molecules does not constitute proof of the validity of the method.

Key words: Capillary permeability, theory of measurements of capillary permeability.

Measurements of capillary solute extraction can be used to estimate capillary permeability. Crone (1961) has proposed that the extraction obtained as the relative difference between the initial areas of the concentration-versus-time curves in the venous blood following injection of a bolus containing the test molecule and reference molecule can be given a simple interpretation of capillary diffusion capacity. Problems related to the measurement and interpretation of this extraction, i.e. the single injection venous up-slope (SIVUS) method, have been discussed (see Crone and Lassen 1970).

Sejrsen (1970) proposed an alternative method of measuring the extraction. It is based on external measurement of the residue function and thus has the interesting feature that it can be used on organs with multiple outlets. Furthermore, the method is much less demanding from an experimental point of view and therefore of considerable potential clinical interest. The method, i.e. the single injection residue function (SIRF) method, has recently been applied to adipose and subcutaneous tissue (Paaske and Levin Nielsen 1976, Paaske 1977a and b). However, Sejrsen did not offer any theoretical justification for the method and it is not obvious what kinds of assumptions that are involved over and above those used in the SIVUS method.

The present paper is an attempt to analyze the situation in some more detail with the object of making explicit the assumptions inherent to the SIRF method.

It has been proposed (Paaske and Levin Nielsen 1976, Paaske 1976, Paaske 1977) that the validity of the SIRF method can be substantiated by the experimental finding that the deduced wash-out function for the transmitted molecules is identical to the observed wash-out action for a reference molecule that cannot pass the capillary membrane, i.e. that

$$\frac{S(t) - F(t)}{S(t_1) - F(t_1)} \text{ is numerically equal to } \frac{S^*(t)}{n} \quad (21)$$

where $S^*(t)$ is the detector signal function for the reference molecules and n is the number of injected reference molecules. We show below that this observation cannot be considered as a strong proof of the validity of the SIRF method.

For any time $t > t_1$ it must be true that

$$\frac{S^*(t)}{n} = \frac{N_R(t_1)}{N_R(t_1)} \frac{N_R(t) + N_{R,v}(t)}{N_R(t_1)} \quad (22)$$

However using (4) and (7)

$$\frac{S(t)}{S(t_1)} \frac{F(t_1)}{F(t_1)} = \frac{N_R(t)}{N_R(t_1)} \frac{N_R(t)}{N_R(t_1) + N_{R,v}(t_1) + N_{R,v}(t)} \frac{N_R(t) + N_{R,v}(t)}{N_R(t_1) + N_{R,v}(t_1) + N_{R,v}(t)} \frac{N_{R,v}(t)}{N_{R,v}(t_1)} \frac{F(t)}{F(t_1)} \quad (23)$$

The right hand sides of (22) and (23) become identical when

$$t > t_0 \quad (24)$$

$$N_{R,v}(t) = N_{R,v}(t_1) = 0 \quad (25)$$

$$N_R(t_1) = 0 \quad (26)$$

$$F(t) = N_{R,v}(t) \quad (27)$$

Thus, if the assumptions are correct, there will be numerical identity between the deduced wash-out curve for the transmitted molecules and the observed wash-out curve for the reference molecules. However as shown by the expressions (22) and (23), an observed numerical identity does not permit the conclusion that the assumptions are correct.

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can be determined for all values of $t \geq t_1$ where t_1 is the time at which $S(t)$ has its maximum value. The proposition is based on the following two assumptions

(i) There exists a time $t = t_1 > t_0$ when all the transmitted molecules have left the detector field. Thus,

$$N(t) = 0 \text{ for } t > t_1 \quad (12)$$

It is assumed that

$$N_d(t) \sim N_e(t) < N_{ex}(t) \text{ for } t > t_1 \quad (13)$$

so that according to (5)

$$N_{ex}(t) \sim S(t), \text{ for } t > t_1 \quad (14)$$

(ii) The form of $S(t)$ for $t > t_1$ can be used, by means of extrapolation back to t_0 , to determine $F(t_1) = N_{ex}(t_1)$

A postulate Sejrsten's postulate is that

$$E = F(t_1)/S(t_1) \quad (15)$$

so that according to (11) and (5),

$$E = N_{ex}(t_1)/N(t_1) + N_d(t_1) + N_{ex}(t_1) + N_e(t_1) \quad (16)$$

Substitution of (6) and (7) into (16) yields

$$E = \frac{N_R(t_1) - N_{R,d}(t_1) - N_{R,e}(t_1) - N_{R,e}(t_1)}{N(t_1) + N_d(t_1) + N_R(t_1) + N_{R,d}(t_1) - N_d(t_1) + N_{R,e}(t_1) + N_{ex}(t_1)} \quad (17)$$

Using (8) (9) and (10) equation (17) becomes identical to (3) when

$$t > t_1 \quad (18)$$

$$N_{R,d}(t_1) \sim N_{R,e}(t_1) \sim 0 \quad (19)$$

$$N(t_1) \sim 0 \quad (20)$$

Comments The physical content of the assumptions required by the SIF method can now be summarized as follows.

The proposition (11) requires that the kinetic behaviour of the molecules in the extravascular space can be deduced from the form of $S(t)$ for $t > t_1$. For example, if the form is monoexponential it follows that the probability of translocation across the capillary wall is independent of the position of the molecules in the extravascular space and that the form of $F(t)$ is monoexponential from t_1 . In any case, a model for the kinetic behaviour of the molecules in the extravascular space must be used to prescribe the form of $F(t) = N_{ex}(t)$ at $t < t_1$.

Assumptions (18), (19) and (20) are statements about the situation at the time when $S(t)$ has its maximum value. The first is that, at this time, all the molecules in the bolus have been either transmitted or extracted. The second is that, at the same time, none of the extracted molecules have as yet left the extravascular space. The third is that at the same time, none of the injected molecules have left the detector field.

Assumption (20) can be confronted directly by experiment, but other assumptions and the proposition (11) cannot be checked by the experimental data.

It has been proposed (Paaske and Levin Nielsen 1976, Paaske 1976, Paaske 1977) that the validity of the SIRF method can be substantiated by the experimental finding that the deduced wash-out function for the transmitted molecules is identical to the observed wash-out function for a reference molecule that cannot pass the capillary membrane, i.e. that

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where $S^*(t)$ is the detector signal function for the reference molecules and n is the number of injected reference molecules. We show below that this observation cannot be considered as a strong proof of the validity of the SIRF method.

For any time $t > t_1$ it must be true that

$$\frac{S(t)}{n} = \frac{N_T(t_1) - N_d(t) + N_{E_v}(t)}{N_T(t_1)} \quad (22)$$

However using (5) and (7)

$$\frac{S(t) - F(t)}{S(t_1) - F(t_1)} = \frac{N_d(t_1) - N_d(t) + N_T(t) + N_{E_v}(t) - N_d(t) + N_{E_v}(t) + N_{E_v}(t) - F(t)}{N_d(t_1) + N_d(t_1) + N_T(t_1) + N_{E_v}(t_1) - N_d(t_1) + N_{E_v}(t_1) + N_{E_v}(t_1) - F(t_1)} \quad (23)$$

The right hand sides of (22) and (23) become identical when

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Thus, if the assumptions are correct, there will be numerical identity between the deduced wash-out curve for the transmitted molecules and the observed wash-out curve for the reference molecules. However as shown by the expressions (22) and (23), an observed numerical identity does not permit the conclusion that the assumptions are correct.

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can be determined for all values of $t > t_0$ where t_0 is the time at which $S(t)$ has its maximum value. The proposition is based on the following two assumptions.

(i) There exists a time $t = t_1 > t_0$ when all the transmitted molecules have left the detector field. Thus,

$$N(t) = 0, \text{ for } t > t_1 \quad (12)$$

It is assumed that

$$N_c(t) \approx N(t) < N_{ex}(t) \text{ for } t > t_1 \quad (13)$$

so that, according to (5)

$$N_{ex}(t) = S(t) \text{ for } t > t_1 \quad (14)$$

(ii) The form of $S(t)$ for $t > t_1$ can be used, by means of extrapolation back to $t = t_0$ to determine $F(t) = N_{ex}(t)$

A postulate Sejrsen's postulate is that

$$E = F(t_0)/S(t_0) \quad (15)$$

so that according to (11) and (5),

$$E = N_{ex}(t_0)/N(t_0) + N_c(t_0) + N_{ex}(t_0) + N_r(t_0) \quad (16)$$

Substitution of (6) and (7) into (16) yields

$$E = \frac{N_r(t_0) - N_{ex}(t_0) - N_{ex}(t_0) - N_{ex}(t_0)}{N_c(t_0) + N_c(t_0) + N_r(t_0) + N_{ex}(t_0) - N(t_0) + N_{ex}(t_0) + N_{ex}(t_0)} \quad (17)$$

Using (8) (9) and (10) equation (17) becomes identical to (3) when

$$t > t_0 \quad (18)$$

$$N_{ex}(t) \approx N_{ex}(t) \approx 0 \quad (19)$$

$$N(t_0) \approx 0 \quad (20)$$

Comments The physical content of the assumptions required by the SIRC method can now be summarized as follows.

The proposition (11) requires that the kinetic behaviour of the molecules in the extravascular space can be deduced from the form of $S(t)$ for $t > t_0$. For example, if the form is monoexponential it follows that the probability of translocation across the capillary wall is independent of the position of the molecules in the extravascular space and that the form of $F(t)$ is monoexponential from t_0 . In any case, a model for the kinetic behaviour of the molecules in the extravascular space must be used to prescribe the form of $F(t) = N_{ex}(t)$ at $t < t_0$.

Assumptions (18) (19) and (20) are statements about the situation at the time when $S(t)$ has its maximum value. The first is that, at this time, all the molecules in the bolus have been either transmitted or extracted. The second is that, at the same time, none of the extracted molecules have as yet left the extravascular space. The third is that at the same time, none of the injected molecules have left the detector field.

Assumption (20) can be confronted directly by experiment, but other assumptions and the proposition (11) cannot be checked by the experimental data.



Fig. 1. Experimental design.

Material and methods

Studies were performed in 6 healthy physically fit-trained volunteers with mean age of 25.8 years (range 23–35). The investigations were carried out on an ambulatory basis. The subjects were instructed not to eat or smoke after 10 o'clock p.m. on the day prior to the investigations, which in all cases took place at 8–11 a.m.

Consent was given by each subject after complete description of the protocol. The study was approved by the Ethical Committee of the Medical Faculty at the Lund University.

Polyethylene catheters PE 160 were placed by means of the Seldinger technique in the brachial artery in brachial vein and the femoral vein and German catheter 7 F in the left renal vein. In 3 of the subjects a Gerbale-Labbe catheter 7 F was inserted into the coronary artery through cubital vein. The position of this catheter was controlled by X-ray. After the insertion of the catheters, the subjects rested in supine position for at least 30 min before blood samples were taken for the determination of the basal catecholamine concentrations.

Physical work consisted of bicycling in supine position for 9 min with work load of 50 W and immediately afterwards another 9 min with load of 150 W (Fig. 1). The bicycle used was an electrically braked variable load ergometer (Ergonomic-Elema). The ECG was recorded continuously during the work test.

In two of the three subjects, in which catheterization of the coronary artery was undertaken, the maximum oxygen uptake ($\dot{V}O_2$, ml/min) was determined. These studies showed that work load of 150 W represents 75 per cent of the maximum working capacity. At work load of 150 W the mean heart rate for all participants was 163 beats/min. Thus, it is evident that work load of 150 W represented relatively hard work for this group of subjects.

Blood samples for the determination of catecholamines were drawn simultaneously from all catheters at rest, immediately prior to work and then during the last 3 min of work at 50 and 150 W respectively (Fig. 1).

Plasma noradrenaline and adrenaline were analysed using double labelling isotope derivative technique (Lapinskas and Portney 1970). In order to exclude that the blood drawn from the left renal vein is contaminated with adrenal vein blood, the concentration of cortisol was determined in the left renal vein and compared to the cortisol concentration at other levels. Cortisol was estimated using fluorimetry (Clark and Rubin 1969).

When applying work load comparable with the heaviest used in the present studies, Lønnegren and Andersen (1962) found an increase in the coronary blood flow from about 250 ml/min at rest to about 1000 ml/min during work. Simultaneously the kidney blood flow decreased from about 1100 ml/min to about 300 ml/min. Using these figures and the formula:

$$AV\text{-difference} = \text{plasma catecholamine concentration} \times \text{plasma flow}$$

we have calculated the net output of catecholamines from the coronary artery and from the kidney in comparison with exercise at the work load of 150 W.

Statistical analysis was performed by Student's *t*-test for paired observations. The values are reported as mean \pm SE and the level of significance taken as $p < 0.05$.

Plasma catecholamine levels in the coronary sinus, the left renal vein and peripheral vessels in healthy males at rest and during exercise

By

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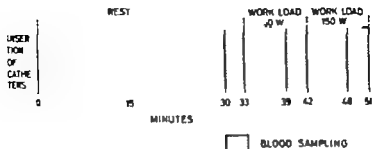
Abstract

MANHEIM, P., H. LECEROF and B. HÖKFELT *Plasma catecholamine levels in the coronary sinus, the left renal vein and peripheral vessels in healthy males at rest and during exercise*
Acta physiol. scand. 1978 104 364-369

Noradrenaline and adrenaline were determined in blood samples from the brachial artery, the brachial vein, the left renal vein and the femoral vein in 6 healthy males (aged 23-35 y). In 3 of the subjects catecholamines were determined also in blood from the coronary sinus. All samples were taken simultaneously in supine position after 30 min of rest and then in connection with exercise in supine position using a bicycle ergometer firstly with work load of 50 W for 9 min and secondly with a work load of 150 W for the same period of time. Under resting conditions the catecholamine levels were about the same at all locations, the mean for noradrenaline being 1.59 nmol/l with a range of $1.30-2.11 \text{ nmol/l}$ and for adrenaline 0.46 nmol/l and $0.23-0.65 \text{ nmol/l}$, respectively. At 50 W the noradrenaline concentration increased significantly in the brachial artery, the left renal vein and the femoral vein, whereas adrenaline increased significantly only in the femoral vein. At 150 W the noradrenaline content increased markedly in all samples, especially in the left renal vein (mean increase 13.02 nmol/l) and the coronary sinus (mean increase 13.06 nmol/l). Adrenaline concentration increased significantly in the brachial artery and the femoral vein. At 150 W the mean net output of noradrenaline as estimated from the calculated flows and actual AV-differences amounted to 2.25 nmol/min from the heart and to 0.36 nmol/min from the kidney.

Key words: Plasma catecholamines, renal, cardiac, exercise

In blood from healthy resting individuals the ratio of noradrenaline to adrenaline is about 5:1 but both absolute and relative amounts vary widely (Engelman and Portnoy 1970). Circulating noradrenaline is believed to originate mainly from the sympathetic nerve endings, adrenaline from the adrenal medulla. The concentration of both these catecholamines in blood increases during exercise (Kotchen *et al* 1971, Christensen and Brandborg 1973, Chodakowska *et al* 1975 and Hansson and Hökfelt 1975). So far only few studies have been performed with the aim of defining the more precise sources of the circulating catecholamines. We have determined the catecholamine concentration in blood at different levels in the arterial and venous vascular system of healthy men at rest and after standardized, light and heavy physical work.



1 Experimental design.

Material and methods

was performed in 6 healthy physically well-trained volunteers with mean age of 25.8 years (range 21–35). The investigations were carried out on an ambulatory basis. The subjects were instructed to not eat or smoke after 10 o'clock p.m. on the day prior to the investigations, which in all cases took place 8–11 a.m.

Informed consent was given by each subject after complete description of the protocol. The study was approved by the Ethical Committee of the Medical Faculty at the Lund University.

Arterial catheters PE 160 are placed by means of the Seldinger technique in the brachial artery, brachial vein and the femoral vein and a femoral catheter 7 F in the left renal vein. In 3 of the subjects a double-lumen catheter 7 F was inserted into the coronary sinus through a cubital vein. The position of the catheter was controlled by X-ray. After the insertion of the catheters, the subjects rested in supine position for at least 30 min before blood samples were taken for the determination of the basal catecholamine concentrations.

Typical work consisted of bicycling in supine position for 9 min with work load of 90 W and immediately afterwards another 9 min with work load of 150 W (Fig. 1). The bicycle used was an electrically load variable load ergometer (Ergonomic-Electro). The ECG was recorded continuously during the work period.

Two of the three subjects, in which cannulation of the coronary sinus was undertaken, the maximum oxygen uptake ($\dot{V}O_2$, max) was determined. These studies showed that work load of 150 W represents 75% of the maximum working capacity. At work load of 150 W the mean heart rate for all participants was 145 beats min⁻¹. Thus, it is evident that work load of 150 W represented relatively hard work for these subjects.

Blood samples for the determination of catecholamines were drawn simultaneously from all catheters at 1 min immediately prior to work and then during the last 3 min of work at 90 and 150 W respectively (Fig. 1).

Plasma noradrenaline and adrenaline were analysed using double labelling isotope derivative techniques (Sjöström and Portier 1970). In order to exclude that the blood drawn from the left renal vein is contaminated with adrenal vein blood, the concentration of cortisol as determined in the left renal vein and compared to the cortisol concentration at other levels. Cortisol was estimated using fluorimetry (Clark & Rabin 1969).

When applying work load comparable with the heaviest used in the present studies, Lunde-Anderson (1967) found an increase in the coronary blood flow from about 250 ml/min at rest to about 1000 ml/min during work. Simultaneously the kidney blood flow decreased from about 1100 ml/min to about 300 ml/min. Using these figures and the formula:

$$AV\text{-difference} = \frac{\text{plasma catecholamine concentration} \times \text{plasma flow}}{\text{arterial concentration}}$$

we have calculated the net output of catecholamines from the coronary sinus and from the kidney in subjects with exercise at the work load of 150 W.

Statistical analysis was performed by Student's *t*-test for paired observations. The values are reported as mean \pm SE and the level of significance taken as $p < 0.05$.

TABLE 1 Noradrenaline, adrenaline and cortisol in blood samples taken from the brachial artery the brachial vein, the left renal vein, the femoral vein and the coronary sinus at rest, respectively at 50 W and 150 W work load.

	Noradrenaline nmol/l			Adrenaline nmol/l			Cortisol nmol/l		
	0	50 W	150 W	0	50 W	150 W	0	50 W	150 W
	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE	mean \pm SE
A. brachialis n=6	1.33 0.22	2.15 0.41	9.54 2.39	0.63 0.43	0.74 0.13	2.17 0.60	260.0 23.6	223.0 ^a 23.2	187.5 26.8
V brachialis n=6	2.11 0.54	2.37 0.53	7.87 1.66	0.50 0.08	0.50 0.09	1.77 0.63	279.9 27.7	260.2 30.8	211.0 ^a 28.8
V renalis n=6	1.75 0.41	2.92 0.43	14.77 4.83	0.41 0.03	0.49 0.05	2.56 1.78	108.0 20.6	166.6 23.4	164.5 26.2
V femoralis n=6	1.30 0.23	2.08 0.39	9.38 2.46	0.23 0.02	0.53 0.08	1.79 ^a 0.55	256.5 27.1	222.6 24.6	200.3 26.5
S. coronarius 3	1.44 0.16	3.06 0.32	14.5 4.65	0.49 0.06	0.67 0.09	1.86 0.39	227.6 42.5	218.3 44.8	185.0 ^a 42.9

Denotes significant alterations compared to basal value at the level of significance where $p < 0.05$ where $p < 0.01$ and where $p < 0.001$

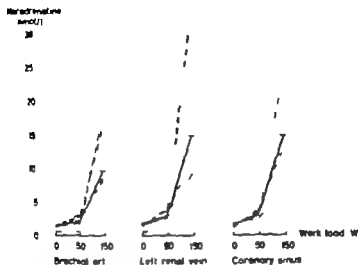


Fig. 2. Noradrenaline in blood taken from the brachial artery, the left renal vein and the coronary sinus at rest, and at 50 W and 150 W work load respectively. Broken lines indicate the values from the individual subjects, solid line indicates the mean values.

Results

The plasma cortisol concentration decreased stepwise at all sites following work and the level in the renal vein did not exceed that found in other locations at the respective times. Thus, there was no significant contamination of blood from the adrenal vein (Table 1).

At rest the concentration of noradrenaline was similar in blood samples from all locations and increased uniformly at 50 W (Table 1). The increase was statistically significant in samples from the brachial artery, the left renal vein and the femoral vein. At 150 W the noradrenaline content increased considerably at all sample sites. However, there was a lower increase of the noradrenaline concentration in blood from the left renal vein ($n = 6$) and from the coronary sinus ($n = 3$) as compared with other locations. Thus, concentration of noradrenaline in the left renal vein significantly exceeded the concentrations in the brachial vein, brachial artery and femoral vein (Fig. 2). In the coronary sinus the noradrenaline concentration at the heavier work load increased to the same level as in the left renal vein, but the increase was not statistically significant, possibly because of the relatively few subjects studied (Fig. 2).

The adrenaline concentration in samples taken at rest was of the same magnitude at all sites and increased in all locations except the brachial vein following a work load of 50 W (Table 1) and at all sites following 150 W.

At 150 W the net output of noradrenaline, calculated as previously mentioned, amounted to 2.25 nmol/minute from the heart and 0.36 nmol/minute from the kidney. Similar calculations revealed no significant addition of noradrenaline from the heart and the kidney under basal conditions and at a work load of 50 W. No other significant AV-differences were reported.

TABLE I Noradrenaline, adrenaline and cortisol: blood samples taken from the brachial artery, the left renal vein, the femoral vein and the coronary sinus at rest, respectively at 50 W and 150 W work load.

	Noradrenaline nmol/l				Adrenaline nmol/l				Cortisol nmol/l			
	0		50 W		150 W		0		50 W		150 W	
	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE
A. brachialis n=6	1.33	0.22	2.15	0.41	9.54	2.39	0.65	0.43	0.74	0.13	2.17*	0.60
V brachialis n=6	2.11	0.54	2.37	0.53	7.87	1.66	0.50	0.08	0.90	0.09	1.77	0.63
V renal vein n=6	1.75	0.41	2.92	0.45	14.77	4.23	0.41	0.03	0.49	0.03	2.56	1.78
V femoralis n=6	1.30	0.23	2.08	0.39	9.38	2.46	0.23	0.02	0.53	0.08	1.79	0.35
S coronary n=3	1.44	0.16	3.06	0.32	14.5	4.65	0.49	0.06	0.67	0.09	1.86	0.39
											227.6	42.5
											218.3	44.8
											187.5	26.8
											211.0*	33.8
											164.5	26.2
											200.3	6.5
											183.0*	42.9

Denotes significant alterations compared to basal value at the level of significance where $p < 0.05$ where $p < 0.01$ and where $p < 0.001$

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Discussion

Yamaguchi (1975) stimulated the sympathetic nerves to the heart in the dog and constantly measured the catecholamines in the blood from the coronary sinus. He concluded that measurements of noradrenaline in blood from the coronary sinus can be used as an indication of the amount of sympathetic neurotransmitter released in heart tissue. Our results seem to support his idea.

Our findings indicate that the heart as well as the kidneys contribute to the increase in noradrenaline in blood occurring in connection with heavy work. It seems likely that the release of noradrenaline in the heart during physical work is even greater than estimated from our figures since part of the noradrenaline released is subject to re-uptake in the neuron, part is taken up by extra neuronal tissues and some is metabolized (Kopin 1977). Moreover it seems probable that some of the noradrenaline transported to the heart from other locations is taken up by heart tissue (Gina and Vane 1968).

Miura *et al* (1976) measured the concentrations of adrenaline and noradrenaline in plasma taken from different parts of the circulatory system in 22 patients with heart disease at rest and following handgrip exercise. According to their calculations the contribution of noradrenaline from the coronary sinus was rather small not only at rest but also during exercise. This difference in their results and ours probably is due to the low work load applied in the experiments of Miura *et al*.

Previously no attempts seem to have been made to calculate the noradrenaline output from the kidney during heavy work. Our results probably reflect considerably increased renal sympathetic activity at heavy work.

In healthy resting individuals Vendsalu (1960) found that the adrenaline concentration was significantly higher in the brachial artery than in the brachial vein, while the opposite was true for noradrenaline. In our study we found no such differences, either at rest or in connection with work. We can offer no definite explanation for the discrepancy between Vendsalu's findings and ours. However it may be pointed out that he investigated a larger number of individuals than we did and that the differences found by Vendsalu, although statistically significant were rather small. Furthermore, Vendsalu used a fluorometric method to measure catecholamines, which is less capable of differentiating between noradrenaline and adrenaline as compared to the method used by us.

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1. Blood values at the end of exercise.

xi	Femoral venous blood			Arterio-venous difference
	pH	PCO ₂ (kPa)	Lactate (mmol/l)	Lactate (mmol/l)
	6.94	10.1	20.6	-2.7
	7.00	9.3	14.1	-1.4
	7.08	8.1	20.3	-2.4

During exercise BD_b increased in arterial blood with the same amount as lactate + pyruvate (1). This equivalence indicates that BD_b during this period accompanies the transport of lactate from the site of production (*i.e.* muscles working at a maximal load) to the blood to tissues utilizing lactate (*i.e.* liver, heart and submaximally exercised muscles). During the early part of recovery the equivalence disappeared, BD_b increased, whereas content of lactate + pyruvate did not. This finding is in agreement with previous observations that after maximal exercise, increase in arterial BD_b is higher than corresponding accumulation of lactate + pyruvate (Bouboys *et al.* 1966, Osnes and Hermansen 1972). The present investigation is the first to demonstrate that increase in hydrogen ions in blood is equivalent to the accumulation of lactate + pyruvate during the exercise and that the discrepancy occurs first during recovery. It has previously been proposed that other acids than lactate (*i.e.* pyruvate + free fatty acids) accumulate in blood and, thus, would explain the excess increase of BD_b (Doll *et al.* 1968). The increase of blood content of free fatty acids after exercise is, however, too small and the time course as described by Keul *et al.* (1969) not consistent with the increase in BD_b . In the present study it has also been shown that accumulation of pyruvate is too small to have any importance.

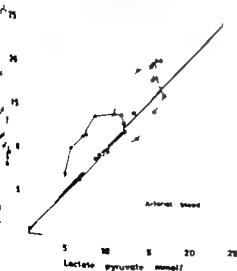


Fig. 1. Base deficit in blood (BD_b) compared with content of lactate + pyruvate in blood from arterial brachial samples. Samples were taken at rest (—), during exercise (O), after 0–3 min recovery (●), and after 7–30 min recovery (□). Values from the same subject are joined together. — subject K, O — subject A, □ — subject C. R. Content of pyruvate is at all times very small (< 0.5 mmol/l).

Acid base balance in blood during exhaustive bicycle exercise and the following recovery period

By

K. SÄLLIN, A. ALVSTRAND, R. BRANDT and E. HULTMAN

Under intense muscular exercise part of the energy is provided anaerobically by lactic acid formation. The appearance of lactate in blood is associated with an increase of the hydrogen ion content. It is of great importance for the understanding of acid-base balance in tissue and blood during lactic acidosis to know whether lactate is transported through the cell membrane at the same rate as the hydrogen ion or not. In the present study this has been investigated by analyses of blood samples taken during exercise and the following recovery period.

Methods

Three healthy male subjects aged 24-29 years participated in the experiment. The nature and purpose of the study was explained to the subjects before their voluntary consent was obtained.

The subjects exercised in sitting position on a electrically braked bicycle ergometer (Siemens-Ele AB, Sweden) at a continuous pedalling rate of 60 rpm. The work load calculated to lead to exhaustion after 6 min (W_{max}) was determined for each subject in a preceding session according to the method of Torvald (1963) and was 275-300 W. Subjects worked 5 min at 100 W and thereafter at W_{max} until exhaustion. Total work time was 10-10.8 min and max pulse rate 170-190. After exercise the subjects rested in the supine position.

Before exercise catheters were inserted in arterial brachials and deep femoral vein. Arterial and venous blood were simultaneously collected at rest during the last 4 min of exercise and during the recovery period. Samples (5 ml) were taken anaerobically in heparinized syringes and were stored in ice bath and analyzed.

Acid-base data in blood (pH, PCO_2 and PO_2 and Hb) were analyzed in an automatic system (Rabometer Copenhagen, Type ABL 1). Base deficit in blood (BD_b) was automatically calculated by the instrument from the measurements.

Immediately after the acid-base measurement, 1 ml of the blood sample was precipitated with 2 ml of 0.65 M perchloric acid. Lactate and pyruvate were analyzed by enzymatic methods on neutralized samples.

Results and Discussion

Blood content of lactate and hydrogen ion increased continuously during the exercise and reached high values at the end of exercise (Table I). The release of lactate from the working muscle continued during the recovery period.

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1. Blood values at the end of exercise.

Femoral venous blood	Arterio-venous difference		
pH	PCO ₂ (kPa)	Lactate (mmol/l)	Lactate (mmol/l)
6.94	10.1	20.6	-2.7
7.00	11.8	14.1	-1.4
7.05	8.1	20.3	-2.4

During exercise BD_b increased in arterial blood with the same amount as lactate + pyruvate (1). This equivalence indicates that H^+ during this period accompanies the transport from the site of production (*i.e.* muscles working at a maximal load) to the blood in tissues utilizing lactate (*i.e.* liver, heart and submaximally exercised muscles). During early part of recovery the equivalence disappeared, BD_b increased, whereas content of pyruvate did not. This finding is in agreement with previous observations that after maximal exercise, increase in arterial BD_b is higher than corresponding accumulation of H^+ (Boekheys *et al.* 1966, Osnes and Hermansen 1972). The present investigation is the first to demonstrate that increase in hydrogen ions in blood is equivalent to the accumulation of lactate + pyruvate during the exercise and that the discrepancy occurs first during recovery. It has previously been proposed that other acids than lactate (*i.e.* pyruvate and free fatty acids) accumulate in blood and, thus, would explain the excess increase of BD_b (Doll *et al.* 1968). The increase of blood content of free fatty acids after exercise is, however, too small and the time course as described by Keul *et al.* 1969 not consistent with the change in BD_b . In the present study it has also been shown that accumulation of pyruvate is small to have any importance.

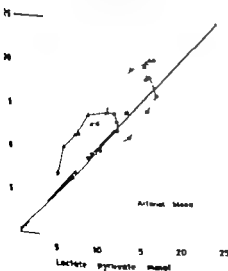


Fig. 1. Base deficit in blood (BD_b) compared with content of lactate + pyruvate in blood from arterial brachialia. Samples were taken at rest (Δ), during exercise (\circ), after 0-2 min recovery (\oplus), and after 7-30 min recovery. A: Values from the same subject are joined together. — subject K, \circ — subject A, \oplus — subject C. R: Content of pyruvate is at all times very small (< 0.5 mmol/l).

Another possible explanation for the changed relation between BD and lactate content is that hydrogen ions are released at a faster rate than lactate from exhausted muscle. This hypothesis is supported by a recent study of muscle metabolism (Sahlin *et al.* 1976) where muscle samples taken during recovery from exhaustive bicycle exercise were analysed for pH and content of lactate-pyruvate. The data indicated that H^+ was transported out of muscle in excess of lactate during the early part of recovery. It has also been shown that electrically stimulated muscle *in vitro* has a faster efflux of H^+ than of lactate during the recovery period (Heisler 1973).

The mechanism for hydrogen ion transport through the muscle cell membrane is unknown. Recent studies (Roos 1975; Mainwood and Worsey-Brown 1975) indicate that some H^+ is transported as undissociated lactic acid. Other transport mechanisms involving movements of ions (*i.e.* H^+ or HCO_3^-) ought to be influenced by the membrane potential.

It has been shown (Harris *et al.* 1976) that there is a rapid resynthesis of creatine phosphate during the first minute of recovery. This resynthesis is associated with a release of hydrogen ions. A changed membrane permeability due to altered membrane potential, in combination with an intracellular hydrogen ion production related to creatine phosphate resynthesis might be the reason for hydrogen ion efflux in excess of lactate during the early part of recovery.

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Local lactate and exhaustion

By

PETER TEACH

well established that with exhaustive exercise lactate formation will take place and maximal levels in active muscles within a few minutes (e.g. Karlsson 1971). Approximately the same lactate concentrations were obtained irrespective of performance times depending to 0.5-10 min. These data were obtained in homogenates of whole muscle fibres and there were indications that lactate gradients existed within the biopsy sample (Karlsson 1971). Recently microdissection technique has been developed which makes it possible to study the lactate distribution pattern within the two main muscle fibre types, fast twitch (FT) and slow twitch (ST) muscle fibres (Easton 1978). The following experiment was undertaken in order to study the distribution of lactate in pools of different muscle fibres from biopsy samples taken after short-term (1-2 min) exercise with an energy need depending to a level exceeding maximal oxygen uptake (approximately 120% of \dot{V}_{O_2} max). Seven males characterized by a high aerobic capacity participated in the study. Age, height, weight, maximal oxygen uptake and muscle fibre type distribution in m. vastus lateralis were: 22 (19-27) yrs, 180 (176-186) cm, 69 (63-77) kg, 66 (61-71) ml $\text{kg}^{-1} \text{min}^{-1}$ and 70-62% fast twitch (FT) fibres, respectively. As observed in earlier studies a positive correlation was present between individual \dot{V}_{O_2} max and per cent ST fibres (e.g. Costill & Hodgson 1976) ($r = 0.80$, $p < 0.05$). The subjects performed bicycle exercise leading to exhaustion in 1.67 (1.05-2.10) min. Inability to maintain a certain pedal frequency (70 rev min^{-1}) was taken as criterion for exhaustion. Blood lactate concentration obtained 3 min after exercise corresponded to 11.7 (8.3-17.3) mmol l^{-1} blood. Lactate concentration in freeze dried, dissected fragments of muscle fibres averaged in FT fibres and ST fibres 25.8 (14.4-35.0) and 18.7 (13.6-26.5) mmol kg^{-1} wet muscle, respectively ($p < 0.05$). The mean FT/ST lactate ratio was 1.4 (1.1-2.3). Lactate concentration, calculated for the whole muscle (Teach *et al.* 1978) averaged 20.8 (13.9-30.5) mmol kg^{-1} wet muscle and was related to the percentage of FT fibres ($r = 0.85$, $p < 0.05$ Fig. 1). A relationship between lactate concentration and performance time was demonstrated (Fig. 2, $p < 0.05$, Fig. 2). In addition, it was noted that subjects with muscles rich in FT fibres had higher performance capacity in the anaerobic bicycle test. These findings indicate higher anaerobic power and capacity in subjects rich in FT fibres, though subjects extreme in that respect were not included in the present study. Higher ability to form lactate might be one contributing factor to anaerobic performance capacity. Our results also indicated that, within one and the same muscle, FT fibres form more

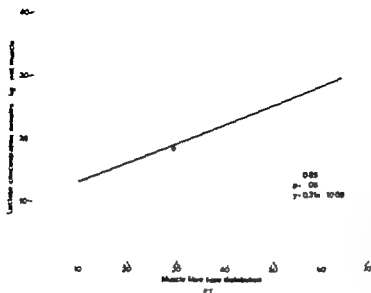


Fig. 1. The relationship between muscle fibre type distribution (FT) and lactate concentration ($n = 7$).

lactate compared to ST fibres. This would also be in accordance with the different metabolic profile of the two main muscle fibre types (e.g. Sjödin 1976, Thorstensson 1978). However, it cannot be excluded that physical conditioning partly will contribute to the FT/ST lactate ratio observed.

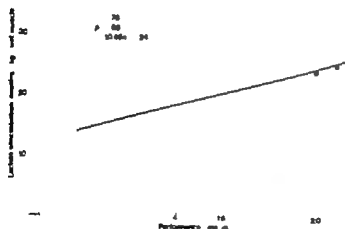


Fig. 2. The relationship between performance time (min) and lactate concentration ($n = 7$).

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Release of neurotensin-like Immunoreactivity (NTLI) from the gut in anaesthetized dogs

By

M. L. MARFORD, GÖRAN NILSSON, ÅKE RÖRGEFUS and SUNE ROSELL

Neurotensin is a tridecapeptide with the sequence <Glu-Leu-Tyr-Glu-Asp-Lys-Pro-Arg-Phe-Tyr-Ile-Leu-OH (Carraway and Leeman 1975). It was first found in the bovine adrenomedulla by Carraway and Leeman (1973). It is noteworthy that in the rat some 85% of the body's neurotensin is located in the gut and particularly in the more distal part of the small bowel (Carraway and Leeman 1976). Immunofluorescence histochemical studies have shown glanular cells in the mucosa of the ileum which react with antibodies against neurotensin (Orl *et al.* 1976, Sundler *et al.* 1977 and Helmstaedt *et al.* 1977). No plausible physiological function has been ascribed to the gut neurotensin but its cellular location suggests a hormonal role. The target tissue for neurotensin in such a hormonal role has not been found. However the peptide given by intravenous infusion in moderate doses elicits inhibition of gastric motility (Andersson *et al.* 1977), suppression of gastric acid secretion (Andersson *et al.* 1976), vasodilatation in the gastrointestinal region and a delayed vasoconstriction in subcutaneous adipose tissue (Rosell *et al.* 1976). A requirement for a hormonal role is that neurotensin is released into the blood. Here we present experimental evidence that NTLI is released from the distal ileum into the blood and into the lumen of the bowel. The antiserum (0-7607) used in the assay of neurotensin was raised in rabbits by injection of neurotensin coupled to bovine serum albumin using 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide HCl. The antigen was injected with Freund's complete adjuvant at 3-8 week intervals. This antiserum reacts with neurotensin (NT), NT(1-12), (Glu)⁹NT and Arg-NT(1-11) but not with NT(4-13) or smaller C-terminal fragments. It shows effectively no crossreactivity with the gastric inhibitory polypeptide (GIP), the vasoactive intestinal peptide (VIP), secretin, cholecystokinin-33, cholecystokinin-39, pancreatic glucagon, substance P, leucine, somatostatin, gastrin-17, gastrin-34 or with trypsinized gastrin-34 which presumably contains the N-terminal heptadecapeptide. Synthetic neurotensin (Beckman) was assayed using the chloramine T method. The assay can measure levels down to 10 pM. To determine whether the NTLI in the blood of the nembutal anaesthetized dog could be derived from the gastro-intestinal tract, blood was collected from carotid artery, femoral vein and a mesenteric vein draining the terminal 20 cm of the ileum. The blood flow in the mesenteric vein was registered continuously by photoelectric drop recorder to be able to measure the net outflow of NTLI from the ileum. The concentrations of NTLI were similar in plasma from the artery and the femoral vein and there was a positive V-A difference across the terminal ileum (Table I). In these experiments a loop of the terminal 20 cm of the ileum was perfused through polyethylene tubes introduced into the lumen with a buffered solution (NaCl 70 mM, KCl

TABLE 1 Neurotensin-like immuno-reactivity ($M \pm S.E.$) (Student's *t*-test).

Source	Concentration pM	V A pM	Release fmol min ⁻¹
Carotid artery	40 \pm 6.7 (8)		
Venous blood draining distal ileum	91 \pm 9.7 (7)	51 \pm 11	233 \pm 46.1
Femoral vein	46 \pm 5.9 (8)		
Perfusate distal ileum	521 \pm 160 (7)		

$p < 0.01$

5 mM CaCl 25 mM Tris-HCl 70 mM pH 7.4) at a rate of 1 ml min⁻¹. The perfusate collected from the distal ileum was found to contain NTLI in all animals. The concentration in the perfusates were generally considerably higher than that in mesenteric venous plasma. The function of NTLI in the lumen is not known. One possibility is that neurotensin secreted into the gut may be absorbed with or without change in passage across the intestinal wall. In 3 dogs 6 nmol neurotensin were instilled into the perfused terminal ileum and blood was sampled from the vein draining the loop. The mean concentration of NTLI rose from a baseline of 66 pM to 470 pM after 3 min. It would appear then, that neurotensin in the lumen contents can be absorbed in a form which is immunoreactive.

These findings have shown that plasma from dogs contain material which reacts with an antibody directed mainly against the N-terminus of neurotensin and that such material is released into the blood by the terminal ileum. NTLI also appears in perfusates of the terminal ileum. The peptide in the lumen may represent part of a pathway for passage of neurotensin into the blood since authentic neurotensin in the fluid seems to be absorbed in an immunoreactive form.

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Insulin production by pancreatic islets of obese-hyperglycemic mice cultured for one week in different glucose concentrations

By

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Abstract

ANDERSSON, A. K. ASPLUND and R. LARKINS. *Insulin production by pancreatic islets of obese-hyperglycemic mice cultured for one week in different glucose concentrations*. Acta physiol. scand. 1978. 104. 377-385.

Islets of pancreatic islets isolated from obese-hyperglycemic mice (gene symbol ob) for one week in different concentrations of glucose (3.3, 5.6 or 16.7 mM) was found to markedly affect the functional behaviour of the islet B-cells. Thus, the insulin content of islets cultured at 3.3 mM glucose (subphysiological or supraphysiological glucose concentrations respectively) was actually reduced. Islets cultured in 5.6 or 16.7 mM glucose displayed normal insulin secretory responses to glucose stimulation. Islets cultured in subnormal glucose concentration (3.3 mM) showed reduced insulin response to glucose stimulation in batch type incubations and also lacked second phase of insulin secretion in short perfusion experiments. The rate of insulin biosynthesis of non-cultured islets was higher than that of islets from their lean siblings but culture for one week in 3.3 mM glucose induced pronounced impairment of the insulin biosynthesis in islets of obese as well as lean mice. The present data indicate that the hyperfunction of the islets of the ob/ob mouse at least in part is reversible phenomenon, suggesting that inherent properties of islet B-cells do not act as "primary" factors in the development of the obese hyperglycemic syndrome.

Key words: ob/ob mice, tissue culture, pancreatic islets, glucose, insulin biosynthesis, insulin release.

The obese-hyperglycemic syndrome of the ob/ob mouse (gene symbol ob) is genetically determined and characterized by overeating, obesity, hyperglycemia, hyperinsulinemia and insulin resistance (Ingalls *et al.* 1950, Blesch *et al.* 1952, Westman 1968). The etiology of the syndrome is unknown. Adipose tissue from lean mice transplanted to obese ob/ob mice increased markedly in size. Similarly adipose tissue of obese mice acquires the same characteristics as the host adipose tissue when grafted into lean mice (Ashwell *et al.* 1976). This would argue against primary alterations of the adipose tissue as determinants of obesity in the ob/ob mice. In determining the sequential manifestation of the metabolic characteristics in young ob/ob mice, hyperinsulinemia, hypoglycemia and obesity have been found to precede the onset of hyperglycemia and insulin resistance (Dubac 1976). Therefore, a

primary role for pancreatic islet disturbances in the development of the obese-hyperphagic syndrome must be considered.

In the present study tissue culture has been employed to evaluate the inherent properties of islets isolated from obese hyperglycemic mice and, in some experiments, from their litter mates. The islets were cultured for one week at different glucose concentrations, their insulin producing capacity being evaluated at the end of the culture period.

Materials and methods

Chemicals. Collagenase type CLS was obtained from Worthington Biochemical Corp. Freehold, N.J., U.S.A. Tissue culture medium (TCM 199 either glucose free or containing 5.6 mM glucose), Hanks solution and calf serum was from SBL, Stockholm, Sweden. 125 I-insulin for the radioimmunoassay of insulin and L-[4,5- 3 H]-leucine were from the Radiochemical Center, Amersham, England. Guinea pig anti-insulin serum (AIS) was purchased from Miles Laboratories Inc., Kankakee, Ill., U.S.A. Cell-activated Sepharose-4B was from Pharmacia Fine Chemicals, Uppsala, Sweden and Solcote 100 Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A. The other chemicals used were of analytical grade.

Animals and islet preparation. Non-inbred mice of both sexes with the obese hyperphagic syndrome (genotype ob/ob, Uppsala colony), 5-7 months old, weighing 50-60 g. or their lean litter mates were used. The animals were fasted overnight before the experiments and they were killed by decapitation. Islets obtained by a modified collagenase method (Howell and Taylor 1968) were either used immediately in short-term experiments or transferred to plastic Petri dishes (NUNC, Roskilde, Denmark) containing tissue culture medium TCM 199 supplemented with 10% calf serum, antibiotics and the glucose concentration was adjusted to 3.3, 5.6 or 16.7 mM. For further details of the culture technique reference should be made to Andersson (1976).

Islet morphology. Cultured islets were fixed in Bouin's solution and embedded in paraffin. Sections 4 μ m thick were stained with hematoxylin-eosin or aldehyde fuchsin-trichrome.

Insulin content and secretion. Groups of 10 freshly isolated or one week cultured islets were washed briefly in Hanks solution and homogenized by sonication for 30 s in 500 μ l acid ethanol and extracted overnight at +4°C. The extracts were stored at -20°C before the insulin assay which was performed by means of a radioimmunoassay procedure (Hedl 1972), separating free and antibody bound insulin by ethanol precipitation and using crystalline mouse insulin as standard.

Batch type incubations of one week cultured islets were performed in glass vials containing 250 μ l of bicarbonate buffer (Krebs and Henseleit 1932) supplemented with 10 mM HEPES, 2 mg/ml bovine albumin and glucose and theophylline as given below. Before the experiment each vial was gassed for 1 min with $O_2 + CO_2$ (95:5). The incubation lasted for 60 min and was performed in a water bath at +37°C with continuous shaking. At the end of the incubation the medium was removed, frozen and stored at -20°C prior to insulin assay.

Islet perfusion. The perfusion technique was adapted from Lacy *et al.* (1972) with minor modifications. Groups of 50 islets, either freshly isolated or cultured for one week, were perfused in several parallel experiments. The medium consisted of the buffer described above except for lower albumin concentration (0.5 mg/ml). The flow rate was adjusted to 1 ml/min. Effluents were collected at 1-min or 5-min intervals and analysed for insulin content.

Insulin biosynthesis. Non-cultured or cultured islets in groups of 10 to 20 were incubated for 120 min in the presence of 100 μ Ci/ml L-[4,5- 3 H]-leucine (spec. act. 58 Ci/mmol). Labelled proinsulin and insulin in the homogenized islets were separated from other islet proteins by means of an immune binding technique described in detail by Berne (1975). One aliquot of the islet homogenate was taken for RNA measurements as described by Kinsane and Robins (1958).

Results

Islet morphology. It was found that the large islets with a diameter exceeding approximately 250 μ m, which can be obtained from the ob/ob mouse had a tendency to develop central necrosis, as evidenced by a dense white spot appearing in the centre of the explant when



Fig. 1 A Benda-Grod and hematoxylin-eosin stained slice of an ob/ob islet cultured for one week at 3.3 mM glucose. The central area of necrosis is surrounded by normal epitheloid islet cells.

observed in the stereo microscope during the culture. Such areas were most frequently observed in islets cultured in 16.7 mM glucose and were very seldom seen in cultured islets isolated from the lean control mice. Fig. 1 shows a light microscopical section of a cultured ob/ob islet with a central area of necrotic cells.

Islet content. As can be seen in Fig. 2 all groups of cultured islets displayed a lower insulin content than the non-cultured ones. The highest value for the cultured islets was obtained for those cultured in 5.6 mM glucose ($p < 0.02$ vs. islets cultured in 16.7 mM glucose, $p < 0.005$ vs. islets cultured in 3.3 mM glucose), which contained about half as much insulin as that found in the freshly isolated islets ($p < 0.01$). The high (16.7 mM) and the low (3.3 mM) cultured islets had an insulin content, which was reduced to about one half of that in the non-cultured islets ($p < 0.001$).

Islet secretion in batch experiments. As can be seen in Fig. 3 the ob/ob islets cultured in 3.3 mM glucose were found to respond to an acute glucose load with a significantly ($p < 0.05$) increased insulin secretion only at the highest glucose concentration tested (27.8 mM). On the other hand, islets cultured for one week in 5.6 mM glucose displayed an increased rate of insulin secretion already at a concentration of 8.4 mM glucose reaching a maximum at 16.7 mM glucose. In a separate series of experiments with the islets cultured in 5.6 mM glucose, theophylline was excluded from the incubation medium used in the short-term expts. Also in this case glucose exerted a stimulatory effect on the insulin release, the maximal secretory rate being almost 10 times higher at 27.8 mM glucose as compared to the basal rate in the total absence of glucose.

The islets cultured in 16.7 mM glucose for one week had a basal secretory rate per islet, which was about four times higher than those recorded for the other two groups of cultured islets ($p < 0.001$). Moreover at all glucose concentrations these high-glucose cultured islets secreted most insulin, although the difference from the islets cultured in 5.6 mM glucose did not reach statistical significance at the two highest concentrations.

Islet secretion in islet perfusion experiments. The release of insulin from cultured islets obtained from obese-hyperglycemic mice during perfusion with 2.8 and 28 mM glucose is shown in Fig. 4. The rate of insulin output at the low glucose concentration was shown to be dependent on the glucose environment during the culture period, the highest prestimulatory

primary role for pancreatic islet disturbances in the development of the obese-hyperphagic syndrome must be considered

In the present study tissue culture has been employed to evaluate the inherent properties of islets isolated from obese-hyperglycemic mice and, in some experiments, from their litter mates. The islets were cultured for one week at different glucose concentrations, their insulin producing capacity being evaluated at the end of the culture period.

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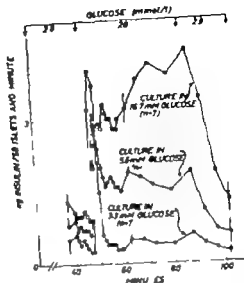


Fig. 4. Fifty ob/ob islets cultured for one week at 3.3 mM glucose concentrations are perfused with 1 or 25 mM glucose during the intervals given by arrows.

Islet biosynthesis. The rates of proinsulin + insulin biosynthesis in non-cultured and shared islets are shown in Table 1. At 3.3 mM glucose, freshly isolated, non-cultured islets obtained from obese mice synthesized significantly more proinsulin + insulin than islets of lean mice. The percentage of total islet radioactivity incorporated into the proinsulin + insulin fraction was also significantly greater in islets of obese animals.

A high glucose concentration (16.7 mM) increased the incorporation into proinsulin + insulin in non-cultured islets from lean as well as obese mice, although the statistical significance was poor for the latter islets ($p = 0.10$). Also at this glucose concentration the islets of the obese mice synthesized more proinsulin + insulin than islets of lean mice. As deduced by the increased percentage, glucose preferentially stimulated incorporation into proinsulin + insulin as compared to other islet proteins in both sets of islets. These increases, however, achieved statistical significance only with islets of lean animals.

Culture for one week at a low glucose concentration (3.3 mM) drastically reduced the biosynthetic activity of islets from both groups of animals. The islets of obese mice, however, still synthesized more proinsulin + insulin than did islets of lean animals during the 1-hour incubation period at 16.7 mM glucose. The biosynthetic rate at 3.3 mM glucose was very low and difficult to differentiate from blank values.

Exposure to 16.7 mM glucose for one week partially eliminated the difference between islets from lean and obese mice, in that the biosynthesis of proinsulin + insulin was similar at low glucose concentrations. These rates were of the same order of magnitude as in non-cultured islets. The percentage of total islet label being incorporated into proinsulin + insulin was, however, still greater in islets of obese mice. A high glucose concentration in the short-term experiments failed to enhance the proinsulin + insulin synthesis in islets of lean mice cultured in high glucose environment. Under the same conditions, islets of obese mice maintained their insulin biosynthetic response to high glucose in absolute terms.

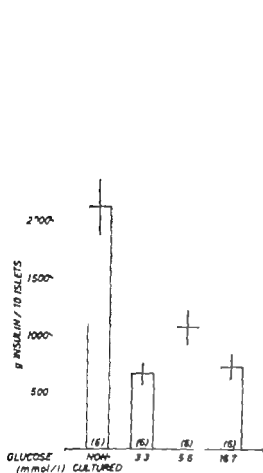


Fig. 2

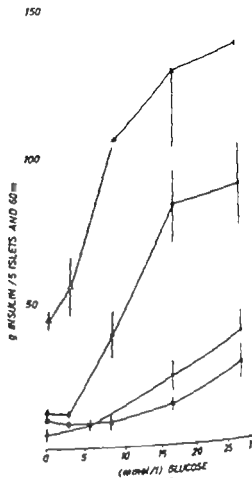


Fig. 3

Fig. 2. Mean insulin content (\pm S.E.) of islets isolated from b/ob mice. The islets were either freshly isolated or cultured for one week at 3 different glucose concentrations.

Fig. 3. Insulin release from one week cultured ob/ob islets as estimated in batch-type experiments, the glucose concentration of the short term incubation media being given at the abscissa. The islets had been cultured either in 3.3 mM glucose (open quadrangles), 5.6 mM glucose (open circles) or 16.7 mM glucose (open triangles). All short term incubations were performed in the presence of 5 mM theophylline except for those denoted by filled circles. The values are given as means \pm S.E. of 4 experiments.

release being observed with islets cultured at 16.7 mM glucose ($p < 0.005$ vs culture in 5.6 mM glucose; $p < 0.001$ versus culture in 3.3 mM glucose). This is in agreement with the batch-type experiments (cf Fig. 3).

Upon raising the glucose concentration of the perfusate to 28 mM the islets responded with a pronounced first phase of insulin release. The magnitude and the temporal characteristics of this early insulin response seemed to be independent of the glucose concentration of the culture medium. On the other hand, the glucose milieu during the culture period was a decisive determinant of the second phase of insulin response to high glucose stimulation. Thus, after culture at 3.3 mM glucose there was a little or no sustained insulin response to 28 mM glucose. Islets cultured at 5.6 as well as 16.7 mM glucose displayed a typical second phase of insulin output, the rate of release per islet being most pronounced after culture at 16.7 mM glucose.

rest during such a prolonged exposure to high glucose to less than 10% (Andersson *et al.* 1974).

The most conspicuous finding in the present investigation was, however, the ability of islets isolated from obese mice to adapt to changes in local environment in a similar way to the adaptation of islets of normal mice to alterations in tissue culture conditions, as previously extensively investigated in our laboratory (Andersson *et al.* 1974; Andersson *et al.* 1976). Thus, after one week in tissue culture, differences in proinsulin + insulin bioassay between obese and lean mice were greatly reduced. In addition, the insulin secretory response to glucose after tissue culture was highly dependent on the glucose concentration of the culture medium. In islets of obese mice, characteristics such as absolute response of insulin response, dose-response relationships and time-dependent profile of hormone secretion were all modified by ambient glucose concentrations during the culture period.

These islets, deprived of the anatomical and metabolic environment that constitutes the obese-hyperglycemic syndrome, thus rapidly adapt to new external conditions. This conclusion does not, however, imply that the pancreatic islets have no central role to play in the pathogenesis of the obese-hyperglycemic syndrome (*cf.* Strautz 1970; Beloff-Chain *et al.* 1973; Larive *et al.* 1977). The hyperinsulinemia in the face of hypoglycemia observed early in the development of the syndrome (Duboc 1976) suggests that factors other than blood glucose are major determinants of B-cell function in young ob/ob mice. Strautz (1970) found that implantation of islets isolated from lean mice into obese mice ameliorates the obese-hyperglycemic syndrome. Parallel observations have been made in New Zealand obese mice (Gates *et al.* 1972). These findings could indicate that normal islets do not react to the metabolic environment in the same way as the islets of developing ob/ob mice. In disagreement with our present findings, this would suggest that there is an intrinsic abnormality in the B-cells of ob/ob mice.

An alternative explanation is that the normal location of the islets is of primary importance for their functional integrity in normal as well as obese mice. This suggestion agrees with the hypothesis of hypothalamic-islet axis that may have an altered functional set-point in at least some forms of obesity (Nagamaski 1972; Asplund 1977). B-cells are clearly under the control of the autonomic nervous system (for review see Woods and Porte 1974), and hypothalamic manipulation causes not only obesity but also acute (Curry and Joy 1974; Rotner *et al.* 1977) as well as long-term (Hustvedt and Lora 1972) effects on insulin secretion. Recent observations by Malaisse-Lagae *et al.* (1977) in C57B1/6J-ob mice, and by Gates and Lazarus (1977) in NZO mice suggest that the hypothalamic abnormality in mice might be due to failure of a feedback signal from the islets, mediated by pancreatic polypeptide. If these results are confirmed, they would explain the observations of Strautz (1970) and Gates *et al.* (1972) cited above, as well as being totally compatible with our own results. Mice suggest normal B-cell adaptation in ob/ob mice when studied devoid of their hypothalamic connexion. Our results argue strongly against inherent properties of the B-cells as "primary" factors responsible for the obese-hyperglycemic syndrome.

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TABLE I Insulin biosynthesis of non-cultured and cultured islets. Islets were isolated either from obese mice or from their lean litter mates. Insulin biosynthesis of freshly isolated islets or islets cultured for one week in 3.3 or 16.7 mM glucose was estimated by measuring the incorporation of tritiated leucine at 3.3 or 16.7 mM glucose for 2 h into both the proinsulin + insulin fraction (PI-I) and TCA precipitable protein fraction of the islet homogenate. The values in column 1 refer to the percentage of the total incorporated radioactivity represented by the PI-I fraction as calculated from each individual observation. DNA was estimated by means of a fluorophotometric method. Means \pm S.E. are given as well as the statistical significance of the difference between lean and obese mice ($^a p < 0.05$ $^b p < 0.001$ $^c p < 0.001$) or between short-term incubation at 3.3 or 16.7 mM glucose ($^d p < 0.01$ $^e p < 0.001$). Numbers of observations are given within parentheses.

Incubation (mM glucose)	3.3			16.7		
	PI-I (cpm $\times 10^{-3}$ /mg DNA 2h)			PI-I (cpm $\times 10^{-3}$ /mg DNA 2h)		
<i>Islet specimen</i>						
Lean non-cultured	23.3 \pm 4.2	19.4 \pm 1.4	(9)	57.3 \pm 7.1 ^b	26.3 \pm 1.6 ^c	(8)
Obese non-cultured	39.7 \pm 13.6	25.0 \pm 2.0	(11)	89.3 \pm 11.6	32.3 \pm 3.0	(8)
Lean cultured in 3.3 mM glucose	—	—		6.8 \pm 2.1	2.6 \pm 0.5	(10)
Obese cultured in 3.3 mM glucose	—	—		13.1 \pm 2.0 ^a	4.4 \pm 0.5 ^a	(11)
Lean cultured in 16.7 mM glucose	39.7 \pm 7.6	9.2 \pm 0.9	(9)	7.3 \pm 5.8	9.4 \pm 1.5	(10)
Obese cultured in 16.7 mM glucose	45.6 \pm 8.2	15.5 \pm 0.8	(7)	73.9 \pm 18.7 ^a	17.2 \pm 1.5 ^a	(10)

Discussion

The tissue culture procedure used in the present series of experiments has been thoroughly evaluated previously and it has proven a useful investigative tool in the study of prolonged effects of various substances on the structure and function of pancreatic islets (Andersson and Hellerström 1976). Thus, by the use of islets isolated from normal mice valuable information on the regulatory mechanisms of insulin production has accumulated (Andersson 1974; Andersson *et al.* 1974). In the present study the tissue culture technique has been used to characterize the possible inherent properties of islets of obese-hyperglycemic mice. Certain aspects of insulin production by cultured islets of obese-hyperglycemic mice has previously been published by other authors (Buitrago *et al.* 1975).

Previously islet hyperplasia (Cameron *et al.* 1972), lower B-cell secretory granule content (Björkman *et al.* 1963) and greater sensitivity of the insulin releasing mechanism to low glucose concentrations (Lavigne *et al.* 1977) have been documented in this strain. The present study adds some further evidence for a modified B-cell function in obese mice when compared to lean mice. Thus, freshly isolated islets of obese animals incorporated more radioactivity into proinsulin + insulin than islets of lean mice at low as well as high glucose concentrations. This was partly due to a larger proportion of the total islet biosynthetic activity being diverted to proinsulin + insulin production in obese animals. Furthermore the insulin content of the islets of the obese mice was reduced to about one third after culture for one week in 16.7 mM glucose, whereas normal mouse islets maintained their insulin

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Fig. 1. Gastrin release (pg/min) into the antral lumen during perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 3-4, and 0.1 M HCl and 0.15 M NaCl pH 6-8, 0.1 M citrate buffer pH 5, 0.1 M citrate buffer pH 3 and 0.1 M HCl. Note that the gastrin release remains relatively unchanged when pH lowered from 6.8-1.2.

	NaCl pH 6.8	NaCl pH 4	HCl pH 1	
	680	500	550	
	1200	1300	1375	
	NaCl pH 6.8	Citrate buffer pH 5	Citrate buffer pH 3	0.1 M HCl
	800	700	750	600
	400		500	400

Methods

Monkeys were anesthetized with chloralose and urethane (50 and 100 mg/kg). Their antra were isolated by making incisions at the antrum-corpora border and at the pylorus. Antral perfusions were performed with various solutions of various pHs via pyloric catheter. The perfusate left the antrum against slight suction (10 cm H₂O) via small cannula inserted into the antral wall. The rate of the perfusion was 2 ml/min. The pH of the outflowing perfusates were recorded.

The following solutions were used: 0.15 M NaCl (pH 6.8 and 4), 0.1 M HCl, 0.15 M NaHCO₃ (pH 8), 0.1 M phosphate buffer pH 7.4, 0.15 M Tris buffer (pH 8), 0.1 M and 0.01 M NaOH (pH 13 and 12), and 0.1 M citrate buffer (pH 3 and 5). The perfusion time varied between 30 and 100 min. After having passed the pylorus, the perfusate was collected in tubes kept on ice. Samples were taken every 10 min and pH was measured and adjusted to 7. After being boiled for 10 min the samples were frozen until assayed for gastrin. The gastrin output into the antral lumen was then calculated from the gastrin concentration of the perfusate and the rate of the perfusion (ml/min).

During the experiments repeated blood samples for gastrin determinations were taken from the femoral vein. Gastrin was determined by radioimmunoassay as described by Nilsson (1975), using antisera 2604 kindly supplied by professor Jens Rehfeld, University of Aarhus, Denmark.

Results

1. Perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 4, 0.1 M citrate buffer pH 3 and 5 and 0.1 M HCl

In these experiments the antrum was successively perfused with solutions of falling pH for 30 min periods. The gastrin release during each perfusion period was calculated based on the values from 3 consecutive 10 min samples. In all experiments the perfusion was started with 0.15 M NaCl pH 6.8 and was finished with 0.1 M HCl. In between the effect of either 0.15 M NaCl pH 4 (-2) or citrate buffer pH 3 and 5 (-2) was tested. As demonstrated in Table I variations of the antral acidity within the pH range 1.2-6.8 was not accompanied by any significant changes in the basal intraluminal gastrin output. Especially notable is the fact that acidification of the antral perfusates from 6.8 down to 1.2 did not result in any significant inhibition of the basal gastrin release.

2. Perfusion with 0.1 M HCl and 0.15 M NaCl

The antrum was initially perfused with 0.1 M HCl (pH 1.2) and then with 0.15 M NaCl (pH 6.8) for 40 min each. The gastrin release per minute into the antral lumen based on 4 consecutive 10 min samples during perfusion, with the different solutions is seen in Table IIa and b.

Effect of intraantral pH on basal gastrin release into the circulation and antral lumen in anesthetized cats

By

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Abstract

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In acute experiments on cat antral pouches were perfused with solutions of different pH (1-13). At antrum passage the gastrin levels in the perfusates were measured with radioimmunoassay and the amount of gastrin released into the antral lumen per minute calculated. The venous gastrin levels were determined concomitantly. Small amount of gastrin (1 000-1 500 pg/min) were released into the antrum during perfusion with 0.1 M HCl. Subsequent perfusion with 0.15 M NaCl (pH 6.8) did not significantly increase the release of gastrin. On the other hand, 0.1 M phosphate buffer (pH 7.4) caused a dramatic augmentation of the gastrin output into the antral lumen (~17 fold). A concomitant increase of peripheral gastrin levels was observed. Also other alkaline solutions such as 0.15 M NaHCO₃ (pH 8), 0.15 M Tris buffer (pH 8) + 0.01 and 0.1 M NaOH (pH 12 and 13) promoted the release of gastrin. It is discussed whether the gastrin release at alkaline pH is induced by the alkaline pH itself or by anions such as HPO₄, HCO₃ and OH⁻. The apparent effect of pH could then be due to the formation of these ions at higher pH.

The inhibitory effect of antral acidification on the gastric acid secretion was first described by Dragstedt *et al* (1951) and by Woodward *et al* (1954 and 1957). They ascribed the reduction of the HCl-secretion to a decreased release of gastrin into the circulation and proposed the existence of an antral pH control of the gastrin release mechanism. Most conclusions, including those mentioned above, concerning the effect of antral pH on gastrin release were based on indirect evidence, the acid secretory responses. Today however it has been directly shown with radioimmunoassay that acidification of the antrum reduces the gastrin release into the circulation caused by different kinds of stimulation, such as sham feeding (Nilsson *et al* 1972) antral perfusion with ACh (Thompson *et al* 1970) or with peptone (Konturek *et al* 1974) etc. However the reports concerning the effects of intraantral pH on the basal gastrin release are less consistent. The aim of the present investigation was therefore to study the influence of intraantral pH on this type of gastrin release. Cats were provided with acute antral pouches, which were then perfused with solutions of various pH. Since gastrin is released both into the antral lumen and into the circulation (Uvnäs-Wallensten and Rehfeld 1976, Uvnäs-Wallensten 1977) antral perfusates as well as blood samples were analyzed for their gastrin content.

a) Gastric release ($\mu\text{g/min}$) into the antral lumen during a) perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 3-4, and 0.1 M HCl and b) 0.15 M NaCl pH 6-8, 0.1 M citrate buffer pH 3, 0.1 M citrate buffer pH 3 and 0.1 M HCl. Note that the gastrin release remains relatively unchanged when pH is lowered from 6.8-1.2.

	NaCl pH 6.8	NaCl pH 4	HCl pH 1	
1 min	600	500	330	
	1200	1300	1375	
2 min	NaCl pH 6.8	Citrate buffer pH 5	Citrate buffer pH 3	0.1 M HCl
	800	700	730	600
	400		500	400

Methods

Animals were anesthetized with chloralose and urethane (30 and 100 mg/kg). Their stomachs were isolated by tying ligatures at the esophagus-corpora border and at the pylorus. Antral perfusions were performed with various solutions of various pH via pyloric catheter. The perfusate left the stomach against slight pressure (10 cm H_2O) via small cannula inserted into the antral wall. The rate of the perfusion was 2 ml/min. The pH of the outflowing perfusates are recorded.

The following solutions were used: 0.15 M NaCl (pH 6.8 and 4), 0.1 M HCl, 0.15 M NaHCO_3 (pH 8), 0.1 M phosphate buffer pH 7.4, 0.15 M Tris buffer (pH 8), 0.1 M and 0.01 M NaOH (pH 13 and 12), and 0.1 M citrate buffer (pH 3 and 5). The perfusion time varied between 30 and 100 min. After having passed the stomach, the perfusate was collected in tubes kept on ice. Samples were taken every 10 min and pH was measured and adjusted to 7. After being boiled for 10 min the samples were frozen until assayed for gastrin. The gastrin output into the antral lumen was then calculated from the gastrin concentration of the perfusates and the rate of the perfusion (ml/min).

During the experiments repeated blood samples for gastrin determinations were taken from the femoral vein. Gastrin was determined by radioimmunoassay as described by Nilsson (1975), using antiserum 2604 kindly supplied by professor Jens Rieckhoff, University of Aarhus, Denmark.

Results

Perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 4, 0.1 M citrate buffer pH 3 and 5 and 0.1 M HCl

In these experiments the stomach was successively perfused with solutions of falling pH for 30 min periods. The gastrin release during each perfusion period was calculated based on the values from 3 consecutive 10 min samples. In all experiments the perfusion was started with 0.15 M NaCl pH 6.8 and was finished with 0.1 M HCl. In between the effect of either 0.15 M NaCl pH 4 ($n=2$) or citrate buffer pH 3 and 5 ($n=2$) was tested. As demonstrated in Table I variations of the antral acidity within the pH range 1.2-6.8 was not accompanied by any significant changes in the basal intraluminal gastrin output. Especially notable is the fact that acidification of the antral perfusates from 6.8 down to 1.2 did not result in any significant inhibition of the basal gastrin release.

2. Perfusion with 0.1 M HCl and 0.15 M NaCl -4

The stomach was initially perfused with 0.1 M HCl (pH 1.2) and then with 0.15 M NaCl (pH 6.8) for 40 min each. The gastrin release per minute into the antral lumen based on 4 consecutive 10 min samples during perfusion, with the different solutions is seen in Table II a and b.

TABLE II Gastrin release (pg/min) into the antral lumen during antral perfusion with 0.1 M HCl (a) and 0.1 M NaCl (b). The gastrin release was determined during 4 consecutive 10 min periods at pH 1 and amounted to ~1 300 pg/min (350-2 125) and ~1 400 pg/min (290-2 500) respectively

TABLE II a. Antral perfusion with 0.1 M HCl.

Cat no	0-10 min	10-20 min	20-30 min	30-40 min	
1	2 000	2 500	2 000	2 000	2 125
2	400	500	500	400	450
3	500	300	300	300	350
4	2 000	2 500	2 000	2 000	2 125
					M 1 262.5

TABLE II b. Antral perfusion with 0.15 M NaCl.

Cat no	40-50 min	50-60 min	60-70 min	70-80 min	
1	2 550	2 400	2 000	2 400	2 337
2	600	300	500	400	500
3	300	200	300	350	290
4	3 000	2 500	2 500	2 000	2 500
					M 1 406

On the average 1 300 pg/min (350-2 125) were released into the antral lumen during perfusion with HCl and 1 400 pg/min (290-2 500) during perfusion with NaCl. Obviously a shift from a strongly acid perfusion solution (0.1 M HCl) to an almost neutral one (0.15 M NaCl) did not increase the intraluminal gastrin secretion. The peripheral gastrin level also remained unchanged.

3 Perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 (n=8)

In this series of experiments, the antrum was consecutively perfused with 0.1 M HCl (pH 1.2) and 0.1 M phosphate buffer (pH 7.4) for 40 min each. The results which are presented in Table III a and b show that ~1 500 pg/min (150-2 625) of gastrin were released into the antral lumen during perfusion with acid, whereas the release during perfusion with phosphate buffer amounted to ~24 000 pg/min (3 675-50 500). The ratio between the gastrin release observed during perfusion with HCl and phosphate buffer was calculated for each cat (Table IV). On the average the release rate increased 17 fold (6-34) when HCl was changed to phosphate buffer pH 7.4. A simultaneous increase of peripheral gastrin levels was observed (Fig. 1).

On perfusion with phosphate buffer gastrin release remained increased for 30-100 min. However if acid was introduced into the antrum while the gastrin release was still elevated, an immediate return towards prestimulatory gastrin levels occurred (Fig. 1) (n=5). The increased gastrin release caused by antral perfusion with phosphate buffer was independent on intact vagal innervation since phosphate buffer also caused a release of gastrin in cats subjected to previous truncal vagotomy (n=2). The pH of the phosphate buffer was adjusted to 3 and 5 by addition of 0.1 M HCl (n=2). No gastrin release was induced by phosphate buffers of pH 3 and 5. 0.1 M phosphate pH 7.4 was diluted to 1, 100 and 1 000 by

TABLE III Gastrin release (pg/min) into the astraal lumen during astraal perfusion with 0.1 M HCl (a) and 0.1 M phosphate buffer pH 7.4 (b). The gastrin release was determined during four consecutive 10 min periods at each pH and averaged to ~1,500 pg/min (150-2,625) and 24,000 (3,675-30,000) respectively

TABLE III Gastrin release into the astraal lumen (pg/min)

	0-10 min	10-20 min	20-30 min	30-40 min	Mean
1	2,800	3,000	3,000	2,500	2,625
2	700	800	900	1,000	820
3	100	200	100	200	150
4	2,900	2,000	2,000	1,500	2,000
5	2,000	1,500	1,000	1,000	1,375
6	3,000	2,500	2,000	1,500	2,250
7	1,800	2,000	1,000	1,500	1,375
8	2,800	1,000	900	900	1,200
					1,900

TABLE IV Gastrin release into the astraal lumen (pg/min)

	40-50 min	50-60 min	60-70 min	70-80 min	Mean
1	40,000	40,000	80,000	42,000	50,500
2	16,000	15,000	9,000	2,000	10,500
3	2,200	4,300	4,400	3,800	3,675
4	6,000	20,000	17,500	22,500	17,000
5	11,000	12,000	7,500	4,000	8,625
6	32,500	40,000	32,500	20,000	41,000
7	17,500	22,500	28,000	15,000	20,750
8	30,000	30,000	30,000	10,000	40,000
					24,000

release of 0.15 M NaCl. The 0.01 and 0.001 M phosphate buffer caused a significant release of gastrin, (10-fold and 2-fold increase respectively) whereas the 0.0001 M solution failed to do so (a, 2).

TABLE V Gastrin release (pg/min) into the astraal lumen during astraal perfusion with 0.1 M HCl-Tri buffer pH 8, n=3 or NaHCO₃ pH 8, n=3

These buffers caused an increase of the gastrin output into the lumen and into the astraal lumen. A representative experiment is illustrated in Fig. 2.

TABLE VI The ratio between the gastrin release into the astraal lumen during perfusion with 0.1 M HCl and with 0.1 M phosphate buffer pH 7.4

Cryst.	Ratio between gastrin release rate during perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4
1	1.11
2	1.12
3	1.23
4	1.19
5	1.16
6	1.18
7	1.15
8	1.33
Mean	1.17

TABLE II Gastrin release (pg/min) into the antral lumen during antral perfusion with 0.1 M HCl (a) and 0.1 M NaCl (b). The gastrin release was determined during 4 consecutive 10 min periods at each pH and amounted to ~1 300 pg/min (350-2 125) and ~1 400 pg/min (290-2 500) respectively.

TABLE II a. Antral perfusion with 0.1 M HCl.

Cat no.	0-10 min	10-20 min	20-30 min	30-40 min	
1	2 000	2 500	2 000	2 000	2 125
2	400	500	500	400	450
3	500	300	300	300	350
4	2 000	2 500	2 000	2 000	2 125
					M 1 262.5

TABLE II b. Antral perfusion with 0.15 M NaCl.

Cat no.	40-50 min	50-60 min	60-70 min	70-80 min	
1	2 550	400	2 000	2 400	337
2	600	500	500	400	500
3	300	200	300	350	290
4	3 000	2 500	2 500	2 000	2 500
					M 1 406

On the average 1 300 pg/min (350-2 125) were released into the antral lumen during perfusion with HCl and 1 400 pg/min (290-2 500) during perfusion with NaCl. Obviously a shift from a strongly acid perfusion solution (0.1 M HCl) to an almost neutral one (0.15 M NaCl) did not increase the intraluminal gastrin secretion. The peripheral gastrin level also remained unchanged.

3 Perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 ($n=8$)

In this series of experiments, the antrum was consecutively perfused with 0.1 M HCl (pH 1.2) and 0.1 M phosphate buffer (pH 7.4) for 40 min each. The results which are presented in Table III a and b show that ~1 500 pg/min (150-2 625) of gastrin were released into the antral lumen during perfusion with acid whereas the release during perfusion with phosphate buffer amounted to ~74 000 pg/min (3 675-50 500). The ratio between the gastrin release observed during perfusion with HCl and phosphate buffer was calculated for each cat (Table IV). On the average the release rate increased 17-fold (6-34) when HCl was changed to phosphate buffer pH 7.4. A simultaneous increase of peripheral gastrin level was observed (Fig. 1).

On perfusion with phosphate buffer gastrin release remained increased for 30-100 min. However if acid was introduced into the antrum while the gastrin release was still elevated, an immediate return towards prestimulatory gastrin levels occurred (Fig. 1) ($n=5$). The increased gastrin release caused by antral perfusion with phosphate buffer was independent on intact vagal innervation since phosphate buffer also caused a release of gastrin in cats subjected to previous truncal vagotomy ($n=2$). The pH of the phosphate buffer was adjusted to 3 and 5 by addition of 0.1 M HCl ($n=2$). No gastrin release was induced by phosphate buffers of pH 3 and 5. 0.1 M phosphate pH 7.4 was diluted to 1/100 and 1/1 000 by

INTRAANTRAL pH ON GASTRIN RELEASE

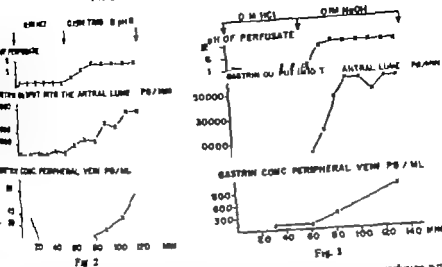


Fig. 2. Gastrin release into the antral lumen (pg/1000) and peripheral gastrin levels during perfusion with 0.15 M HCl (60 min) and 0.15 M Tris buffer pH 8 (70 min). Note the parallel increase of intraluminal gastrin release and peripheral gastrin levels in response to antral perfusion with Tris buffer.

Fig. 3. Gastrin release into the antral lumen (pg/1000), and peripheral gastrin levels (pg/ml) during perfusion with 0.1 M HCl (60 min) and 0.1 M NaOH (80 min). Both the intraluminal output of gastrin and the peripheral gastrin levels increased dramatically in response to perfusion with NaOH.

and secretion increased manyfold in dogs with transplanted antra. Addition of acid to the transplanted antra inhibited the gastric acid secretion. The basal release of gastrin was assumed to be controlled by the antral pH, the release being depressed by acidification. It should be stressed that gastrin measurements could not be performed at this time. Furthermore, the antra transplanted to the duodenum and the colon might have been exposed to gastrin releasing agents present in the intestinal contents not present in an empty antrum *in situ*.

Our results show that in the anesthetized cat basal gastrin release is not appreciably affected by changes of pH in the range of 1-7. They agree with those of McLaughlin *et al.* (1975) who found no difference in the output of gastrin into the portal vein of cats during antral perfusion with 0.15 M NaCl at pH 1 and 8. In contrast to basal gastrin release stimulated gastrin release is efficiently inhibited by antral acidification.

Thus in the cat during antral perfusion with HCl the gastrin release responses induced by electrical vagal stimulation were only 30% of those induced by an identical stimulation during antral perfusion with 0.15 M NaCl (Uvnäs-Wallensten *et al.* 1977).

By which mechanisms does antral perfusion with phosphate buffer and solutions of carbonate and NaOH stimulate the release of gastrin? The gastrin release caused by phosphate buffer pH 7.4 was sometimes enormous (30-fold increase) indicating that phosphate ions might be specifically involved in the release activation. To study this possibility phosphate buffers of lower pH were administered into the antrum. No gastrin release as induced by phosphate buffers of pH 3 or 5 whereas large amounts were released when their pH was increased to 7. Below pH 6 $H_2PO_4^-$ is the dominating anion whereas HPO_4^{2-} predominates around pH 7 indicating that HPO_4^{2-} ions may be the gastrin releasing

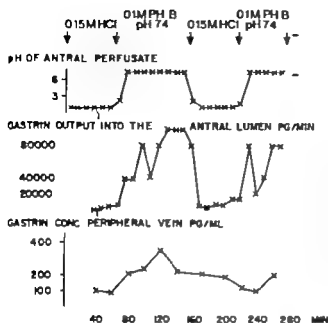


Fig. 1 Gastrin release into the antral lumen (pg/min), and peripheral plasma levels (pg/ml) during antral perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 during two consecutive 80 min periods. Intraluminal gastrin release as well as peripheral gastrin levels increased during perfusion with phosphate buffer. In this experiment the intraluminal release of gastrin lasted as long as the perfusions with phosphate buffer were performed, but decreased as soon as HCl was introduced into the antral pouch.

5 Perfusion with 0.1 M HCl 0.01 or 0.1 M NaOH $n=3$

0.01 M NaOH (pH 12) caused only a slight (two-fold) increase of the gastrin release into the peripheral blood and into the lumen, whereas 0.1 M NaOH (pH 13) released large amounts of gastrin both into the lumen (20-fold increase) and into the circulation. A typical experiment is represented in Fig. 3.

Discussion

The aim of the present investigation was to study the effect of intra-antral pH on basal gastrin release into the antral lumen and into the circulation. The intraluminal gastrin secretion has been described in detail elsewhere (Uvnäs-Wallensten 1977).

The data in this communication show that there is no simple relationship between intra-antral pH and the basal release of gastrin. It was found that even during antral perfusion with 0.1 M HCl gastrin was released into the antral lumen (~ 1500 pg/min). During a preceding as well as during a subsequent perfusion with 0.15 M NaCl (pH 6.8) similar amounts of gastrin were secreted into the lumen. Nor did perfusion with citrate buffer (pH 3 and 5) or with 0.15 M NaCl pH 4 subsequent to perfusion with 0.1 M HCl cause any appreciable increase of the gastrin output. These results indicate that within an intra-antral pH range of 1–7 basal gastrin release is uninfluenced by variations in pH.

On the other hand, when the antrum was perfused with slightly alkaline buffer solutions such as 0.1 M phosphate buffer pH 7.4 sodium bicarbonate or Tris buffer increased amounts of gastrin were released not only into the antral lumen, but also into the circulation—as evidenced by increased peripheral gastrin levels. Perfusion with 0.01 and 0.1 M NaOH also increased the release of gastrin.

Dragstedt and Woodward and their colleagues (1951, 1954 and 1957) studied the effect of pH on the rate of gastric acid secretion in Pavlov pouch dogs, with the antrum in situ, and after its transplantation to the duodenum or colon. They found that the

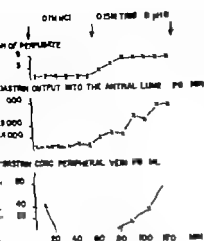


Fig. 2

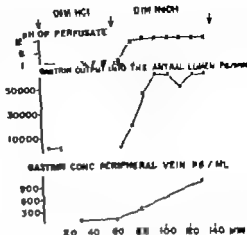


Fig. 3

Fig. 2. Gastrin release into the antral lumen (pg/min) and peripheral gastrin levels during perfusion with 0.15 M HCl (60 min) and 0.15 M Tis buffer pH 7.4 (60 min). Note the parallel increase of intraluminal gastric release and peripheral gastrin levels in response to antral perfusion with Tis buffer.

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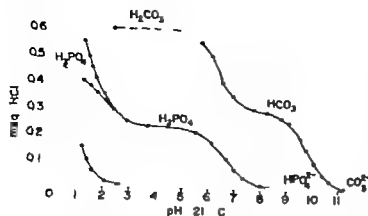


Fig. 4 Titration curves for Na_2CO_3 and Na_2HPO_4 with 5 M HCl. Titration with 200 ml 0.196 M Na_2CO_3 . Titration with 200 ml 0.114 M Na_2HPO_4 . Titration with 200 ml H_2O . The lower titration curve for Na_2HPO_4 demonstrates net values, i.e. total equivalents obtained by titration with 2 ml H_2O (the lowest curve).

factor (Fig. 4). Also perfusion with 0.1 M NaHCO_3 and 0.1 M NaOH released gastrin, whereas perfusion with NaCl pH 8 was without effect. These data indicate that in addition to HPO_4^{2-} , HCO_3^- ions and OH^- ions may stimulate the release of gastrin. Titration curves for Na_2CO_3 and Na_2HPO_4 are presented in Fig. 4.

It should be stressed however that we cannot exclude that the increased gastrin release could be exerted by alkaline pH itself. The stronger buffering capacity of phosphate buffer around pH 7 could in that case explain why antral perfusion with this buffer seemed to release more gastrin than other solutions of the same pH.

However if the gastrin release is induced by anions such as HPO_4^{2-} , HCO_3^- and OH^- where do these ions exert their stimulating effect? Since the antral G-cells have an apical part that projects into the lumen, receptors on the luminal cell surface might be activated. It is also possible that the anions exert their effects intracellularly since they all penetrate cell membranes.

Is the gastrin release promoted by anions such as HPO_4^{2-} , HCO_3^- and OH^- of any physiological significance? It is a well known fact that aromatic amino acids and small peptides are only poor gastrin releasers (Debas *et al.* 1974). In the present experiments even 0.001 M solutions of phosphate buffer stimulated the release of gastrin. Food (e.g. meat and fish) contains substantial amounts of phosphate, the concentration of phosphate ions in e.g. commercial meat broth being 0.005 M. These ions may well contribute to the gastrin release caused by a meal. In this context observations made by Öbrink (1954) are of interest. He found that the gastric secretion in dogs, caused by a bone dust - meat mixture-test meal, could be solely attributed to its phosphate content. The effect might have been mediated via a release of gastrin.

The gastrin release induced by anions such as HCO_3^- might play a role under certain pathological conditions. In the intact empty stomach the pH in the antrum content is controlled by two factors, the alkaline bicarbonate secretion emanating from epithelial cells of the antral mucosa (Grossman 1959; McLeay and Titchen 1975; Flemström 1977) and the HCl secretion emanating from the parietal cells of the corpus. Normally the acid secretion dominates quantitatively rendering the pH of the antral pH more or less acidic. In the absence of HCl secretion the situation may be different. When no acid neutralizes the antral alkaline secretion the pH of the mucosa will rise to 7-8. Thus in patients with atrophic gastritis, which have an intact antral mucosa, the pH may rise to ~8. The

lation is in fact quite similar to that in the present experimental studies in which the isolated antrum is perfused with phosphate or bicarbonate buffers. It is thus possible that the high gastrin plasma levels observed in patients with achlorhydria (McGulgan and Roden 1970 Yalow and Berson 1970, Ganguli *et al* 1971 Stadil and Rehfeld 1973 and Waldenström 1975) may in part be due to an intense activation of the gastrin release caused by endogenous secretion of bicarbonate.

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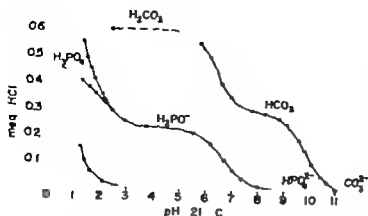


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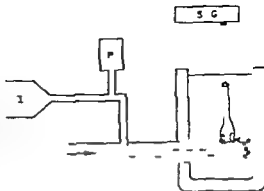


Fig. 1. Experimental set-up for recording of longitudinal tension and resistance to flow through the isolated, perfused bladder-urethra junction. 1, infusion pump; P, pressure manometer; 3-G, strain-gauge transducer. For further details, see text. At the arrow the organ bath supplied with pre-heated, oxygenated Krebs solution.

not only on resistance to flow through the practically closed urethra, but also on longitudinal tension. The aim of the present study was to investigate, by use of this technique, the effects of scrytholine, norendrenaline, and prostaglandins on resistance to flow in the isolated, human fetal urethra. In addition, the occurrence of adrenergic nerve terminals in the fetal bladder bladder neck, and urethra were investigated by means of the fluorescence method of Falck and Hillarp.

Materials and methods

Tissue specimens

Fetal bladder and urethral tissue was obtained as legal interruptions of pregnancy performed by hysterotomy. The estimated gestational age of the fetuses ranged from 16 to 22 weeks. Nine fetuses, 4 female and 5 male, were investigated.

Preparation and mounting

Approximately 10 mm of the urethra attached to the bladder was carefully dissected. The bladder base and small part of the bladder wall were allowed to remain, and were tied to FE 190 catheter. The bladder, all tissue was used to secure the position of the catheter whose tip was placed just before the internal urethral orifice, without stretching or occluding this structure. Through the catheter, which was connected to perfusion pump (Brow perfuser), the bladder neck and proximal urethra were perfused at constant rate of 2 ml/min with pre-heated (37°C) Krebs solution. The recorded resistance to flow was carried largely by the urethra (Persson and Andersson 1976). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.6, CaCl₂ 2.3, MgSO₄ 1.15, NaHCO₃ 24.9, KH₂PO₄ 1.15, glucose 5.5, pH 7.4.

The preparation was mounted in thermostatically controlled 30 ml organ bath (Fig. 1) containing Krebs solution (37°C) bubbled with 95% O₂ and 5% CO₂. The distal end of the urethra was, without occluding the lumen, tied to strain-gauge transducer (Statham Ft 83) for recording of longitudinal isometric tension changes (Fig. 1). Tissue recordings that reflected changes in the urethral smooth muscle tone with minor contribution of the bladder neck (cf Persson and Andersson 1976). Basal tension was set at 3–5 mN depending on the size of the preparations.

Occurrence muscle strips were dissected into preparations being approximately 1 mm wide, 5 mm long and 0.5 mm thick. The apex and the trigonal area of the bladder were cut off and the remaining ring of detrusor muscle was mounted as such, or as longitudinal strip after being cut open.

Histochemical analysis

Tissue specimens for histochemical study were taken from 4 of the fetuses, 2 male and 2 female. Along the longitudinal urethra, the urinary bladder and the urethra were cut into pieces, equal in length and comprising the whole circumference of the respective organs. The tissue pieces were quickly frozen in mixture of propane and propylene at the temperature of liquid nitrogen, freeze-dried and further processed for the Falck-Hillarp technique to demonstrate adrenergic nerves (cf Bjorklund *et al.* 1972).

From the Department of Clinical Pharmacology Institute of Pharmacology University of Århus, Denmark, the Research and Development Laboratories, AB Draco, Lund, the Institute of Anatomy and Histology University of Lund, and the Department of Obstetrics and Gynecology University Hospital of Malmö, Sweden

Effects of acetylcholine, noradrenaline, and prostaglandins on the isolated, perfused human fetal urethra

By

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Abstract

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Preparations of the isolated bladder-urethral junction obtained from human fetuses at mid-gestation were suspended in Krebs solution (37°C), and the urethral lumen was perfused at a low rate (2 ml/h). Resistance to flow and changes in longitudinal tension were recorded. In additional experiments, isometric tension changes were recorded in isolated detrusor strips. The occurrence of adrenergic nerve terminals was investigated by the fluorescence technique of Falck and Hjärup. Acetylcholine (0.1-1.0 µg/ml) produced concentration-related increase in longitudinal tension and in resistance to flow; the effects were unaffected by phenoxybenzamine (0.1 µg/ml), but completely inhibited by atropine (0.1 µg/ml). The effect on tension was more marked than that on resistance to flow. Noradrenaline (0.005-1.0 µg/ml) also produced concentration-related increase in longitudinal tension and in resistance to flow; the effects were abolished by phenoxybenzamine (0.1 µg/ml). The amine had a more pronounced effect on resistance to flow than on longitudinal tension. Prostaglandin $F_{2\alpha}$ (0.1-0.5 µg/ml) increased longitudinal tension and resistance to flow; the effects were not antagonized by atropine (0.1 µg/ml) or by phenoxybenzamine (0.1 µg/ml). In preparations contracted by noradrenaline or acetylcholine, prostaglandins E_1 and E_2 (0.02-0.2 µg/ml) decreased resistance to flow and longitudinal tension. Acetylcholine (0.02-0.2 µg/ml) and prostaglandin $F_{2\alpha}$ (0.0-0.5 µg/ml) contracted, noradrenaline (0.05-0.5 µg/ml) relaxed, and prostaglandin E_1 and E_2 (0.1-5.0 µg/ml) had no consistent effects on the detrusor strips. Histochemically no adrenergic nerve terminals could be demonstrated in the detrusor, bladder neck or urethra.

In isolated strips of smooth muscle from the human detrusor, bladder neck, and urethra, contracting and relaxant effects of drugs acting on cholinergic and adrenoceptors (Todd and Mack 1969, Nergårdh and Boréus 1972, Awad *et al.* 1974, Calne *et al.* 1975, Sundin *et al.* 1976, Ek *et al.* 1977b) and of prostaglandins (Abrams and Feneley 1976, Bultrude *et al.* 1976, Andersson *et al.* 1977) have been demonstrated. However, an *in vitro* analysis of such effects on the resistance to flow through the human urethra has not been possible.

In a previous investigation on the isolated bladder-urethral junction of cats and guinea pigs (Persson and Andersson 1976), a perfusion technique was used, providing information

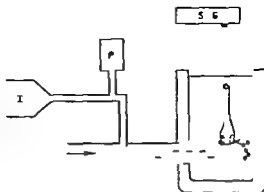


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Detrusor muscle strips were dissected into preparations being approximately 1 mm wide, 5 mm long and 0.5 mm thick. The apex and the trigonal area of the bladder were cut off and the remaining ring of detrusor muscle was mounted as such, or as longitudinal strip after being cut open.

Fluorescence analysis

Tissue specimens for histochemical study were taken from 4 of the fetuses, 2 male and 2 female. Along the longitudinal extremities, the urinary bladders and the urethras were cut into pieces, equal in length and comprising the whole circumference of the respective organs. The tissue pieces were quickly frozen in sections of propene and propylene at the temperature of liquid nitrogen, freeze-dried and further processed for the Falck-Hillarp technique to demonstrate adrenergic nerves (cf. Björklund *et al.* 1972).

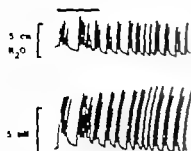


Fig. 2. Spontaneous activity of the fetal bladder-urethral junction. Rhythmic changes in isometric tension (lower tracing) were accompanied by corresponding fluctuations in resistance to flow (upper tracing). Horizontal marker: 4 mm.

From one male fetus, tissue pieces were after the freeze-drying, processed for the immunohistochemical demonstration of peptidergic (VIP: vasoactive intestinal polypeptide) nerves as described by Larsson *et al.* (1976).

Drugs

Drugs used were (–) noradrenaline bitartrate (+) isoprenaline hydrochloride (Sigma Chemical Company USA), phenoxybenzamine hydrochloride (Smith, Kline and French UK), acetylcholine chloride (Calbiochem, USA), tropine sulphate (Pharmacopoea Nordica), prostaglandin E_2 , prostaglandin E_3 (AB ASTRA, Sweden). The drugs were injected directly into the bath. Concentrations given are final bath concentration of active substance.

Results

The perfused fetal human urethra showed a good viability *in vitro*. During the first hour after mounting, the preparations developed a spontaneous contractile activity. Rhythmic changes in isometric tension were accompanied by corresponding fluctuations in resistance to flow (Fig. 2). The spontaneous activity decreased successively and lasted for 1–3 h. Basal tension was stable during the experiment. The basal resistance to flow was also stable, and varied between preparations from 5 to 12 cm H₂O.

Acetylcholine (0.1–1.0 μ g/ml) produced a concentration-related increase in approximate longitudinal tension and in resistance to flow. Particularly at high concentrations, the drug induced a marked rhythmic contractile activity (Fig. 3). In three of the five preparations investigated, marked effects on tension were accompanied by less conspicuous effects on resistance to flow. The effects of acetylcholine were not inhibited by phenoxybenzamine (0.1 μ g/ml), but were completely prevented if atropine (0.1 μ g/ml) was present in the bath (Fig. 3).

In four out of five preparations, noradrenaline (0.005–1.0 μ g/ml) produced a concentration-related increase in longitudinal tension and in resistance to flow (Fig. 4). Noradrenaline also produced a rhythmic contractile activity. The effects were more sustained than those produced by acetylcholine. It was consistently observed that noradrenaline augmented resistance to flow more than it increased longitudinal tension.

Phenoxybenzamine (0.1 μ g/ml) left in contact with the preparation for 20 min, inhibited the noradrenaline effects. In one preparation, no contracting effect of noradrenaline was observed. However, the drug had a slight relaxant action when the preparation was contracted by acetylcholine.

In three preparations contracted by either acetylcholine or noradrenaline, the effects of prostaglandins E_1 (PGE₁) and E_3 (PGE₃) 0.02–0.2 μ g/ml, were

Consistent

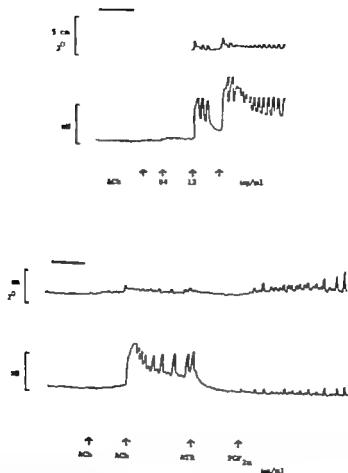


Fig. 1. Above: Effect of cumulative concentrations of acetylcholine (ACh) on longitudinal tension (lower tracing) and on resistance to flow (upper tracing) in an isolated bladder-urethral preparation. Below: Tracing as above. The effects of acetylcholine are abolished when atropine (ATR) is added to the bath. Atropine does not prevent the contractile effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Horizontal marker: 4 min.

Inhibitory effects, i.e., a decrease in resistance to flow and in longitudinal tension, were produced by PGE₂ (Fig. 4). PGE₂ mimicked the actions of PGE₁, except in one preparation, where no effect was registered.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 0.1–0.5 μ g/ml, contracted the preparations, increasing the resistance to flow and longitudinal tension (Fig. 3). The effects of $PGF_{2\alpha}$ were not antagonized by atropine (0.1 μ g/ml) and/or phenoxybenzamine (0.1 μ g/ml).

The detrusor strips ($n = 5$) exhibited spontaneous contractile activity which was present during the first 2 h after mounting. Acetylcholine (0.02–1.0 μ g/ml) and $PGF_{2\alpha}$ (0.1–0.5 μ g/ml) consistently contracted the detrusor preparations and produced a rhythmic contractile activity (Fig. 5). Noradrenaline (0.05–0.5 μ g/ml) inhibited spontaneous (Fig. 5), as did as drug-induced activity PGE₁ and PGE₂ (0.1–5.0 μ g/ml) were without consistent effects on the detrusor preparations.

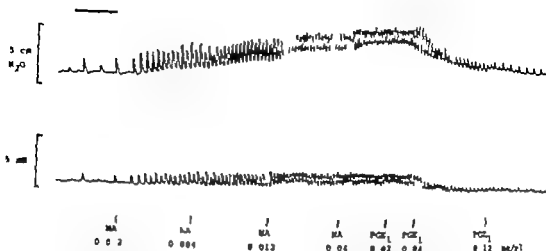


Fig. 4. Cumulating concentrations of noradrenaline (NA) increase resistance to flow (upper tracing) and longitudinal tension (lower tracing) in the bladder-urethral preparation. Prostaglandin E (PGE₂) added in the presence of noradrenaline produces inhibitory effects. Horizontal marker: 4 sec.

Histochemical analysis

Despite serial sectioning, no specific fluorescence for adrenergic nerves or adrenergic ganglion cells could be demonstrated in any part of the urinary bladder and urethra (including the trigonal area and bladder neck), neither in the male nor in the female fetuses. Further, it was not possible in these organs to demonstrate the immunochemical presence of VIP nerves or VIP ganglion cells.

Discussion

Several previous studies have focused on the distribution of adreno- and cholinergic receptors in the human bladder, bladder neck, and urethra. It has been clearly demonstrated that the detrusor and the bladder neck are contracted by cholinergic stimulating drugs, and that this effect can be blocked by atropine (Todd and Mack 1969; Caine *et al.* 1975). It is also generally agreed that the bladder neck contains mainly contraction-mediating α -adrenoceptors, whereas the detrusor has a predominance of relaxation-mediating β -adrenoceptors (Todd and Mack 1969; Nergårdh and Boréus 1972; Awad *et al.* 1974; Sundin *et al.* 1977).

Ek *et al.* (1977b), demonstrated that preparations from all parts of the isolated human urethra, including the urethro-vesical junction, were contracted by noradrenaline in a concentration-related way. Acetylcholine consistently contracted the urethro-vesical junction, but in all other parts of the urethra the sensitivity to the drug was low and often no effects were observed. These *in vitro* results were in agreement with investigations *in vivo*, showing that the intra-urethral pressure can be increased by α -adrenoceptor stimulating drugs (Schreiter *et al.* 1976; Ek *et al.* 1977c), and decreased by α -adrenoceptor blocking agents (Donker *et al.* 1972; Awad *et al.* 1976; Whitfield *et al.* 1976; Ek *et al.* 1977c; Sundin *et al.* 1977). Further, they were consonant with the findings that the *in vivo*

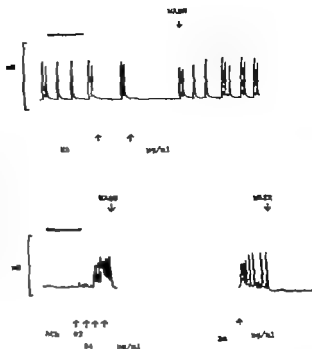


Fig. 5 Upper tracing: Noradrenaline (NA) inhibits the spontaneously active fetal bladder strip. After wash out the contractile activity ceases. Lower tracing: Acetylcholine (ACh) and prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$) contract and alter rhythmic activity in the bladder strip preparation. Horizontal marker 4 mm.

effect of cholinergic stimulation by bethanecol on the urethral closure pressure profile was negligible (Ek *et al.* 1977 c), and that cholinergic blockade by atropine had no effect on this parameter (Ulmsten and Andersson 1977).

In the investigations on the isolated human urethra, (Ek *et al.* 1977 b), the activity of the circular muscle layer was recorded. The technique used in a previous study on the isolated, perfused urethra of cat and guinea-pig (Persson and Andersson 1976) made it possible to measure simultaneously circular muscle activity as reflected by resistance to flow and longitudinal muscle activity registered as changes in isometric tension. Using this technique, it could be shown that in cat and guinea-pig, acetylcholine had less effect on resistance to flow than on longitudinal tension. Furthermore, noradrenaline was more effective than acetylcholine in contracting the circular muscle of the urethra (Persson and Andersson 1976).

The present results agree well with those obtained with the perfused animal urethra. It was found that noradrenaline contracted the isolated human fetal urethra in a concentration-related manner by increasing both resistance to flow and longitudinal tension. The effect on resistance to flow was the more pronounced, probably reflecting a more marked effect on the circular than on the longitudinal muscle layer. Acetylcholine also had a concentration-related contracting action. The effect was less well sustained than that produced by noradrenaline, and longitudinal tension was often more affected than resistance to flow. This suggests that acetylcholine had a more pronounced effect on the longitudinal muscle layer than on the circular

Thus, the results seem to give further support to the view that acetylcholine is of less importance than noradrenaline for maintaining urethral wall tension in man, as this is probably determined mainly by activity in the circular muscle layer.

It was previously shown that prostaglandins E_1 and E_2 decreased resistance to flow through the isolated perfused feline urethra (Persson 1976). *In vitro* these prostaglandins contracted the bladder but relaxed the urethral smooth muscle of human adults (Andersson *et al* 1977). The effects could be reproduced *in vitro* locally applied prostaglandin E_2 increased the intravesical pressure and decreased the intraurethral pressure in women (Andersson *et al* 1978). The present study showed that prostaglandins E_1 and E_2 had no consistent effects on the detrusor preparations, but decreased resistance to flow and longitudinal tension also in the isolated, human fetal urethra.

Adrenergic nerve terminals have been demonstrated in the human detrusor bladder neck and urethra of adults (Mobley *et al* 1966, Sundin *et al* 1977, Ek *et al* 1977a). It was found that in the detrusor and urethra, the smooth muscle cells had a scanty supply of adrenergic nerves, but in the trigone area, the adrenergic nerves were abundant. Gosling and Drue (1976), and Nordling and Christensen (1977) found a very sparse noradrenergic nerve supply in "non-neurogenic human bladders" and the latter authors concluded that this innervation hardly could be of physiological significance. In the present study no adrenergic nerve terminals could be demonstrated, neither in the bladder and urethra, nor in the trigone area. This might be explained by a slow development together with a scanty occurrence of such nerve terminals in the investigated structures.

Despite the absence of adrenergic nerves, well developed responses to noradrenaline were demonstrated. Thus, the distribution and function of adrenoceptors did not seem to be correlated to the occurrence of adrenergic nerve terminals. This is in agreement with the results of other investigators (see, Pappano 1977). In the human fetal heart adrenergic nerve terminals cannot be demonstrated histochemically before the 12th week of gestation (Kanerva *et al* 1974) but a well developed response to adrenaline was found already in the 9th gestational week (Gennser and Nilsson 1970).

Recently the occurrence of nerves containing vasoactive intestinal peptide (VIP) was shown in the genito-urinary tract of pigs, cats, rats, and mice (Alm *et al* 1977, Larsson *et al* 1977a, b). No such nerves were found in the fetus examined in the present study. However, as judged from preliminary results, VIP nerves can be demonstrated in the adult human urinary bladder (Alm unpublished observations).

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Metabolic and cardiovascular responses to prolonged noradrenaline load and their antagonism by beta blockade in the rat

By

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Abstract

HARRI M. N. E. *Metabolic and cardiovascular responses to prolonged noradrenaline load and their antagonism by beta blockade in the rat* Acta physiol. scand. 1978. 104 402-414.

Rats were given daily injections of noradrenaline (0.5 mg/kg), alprenolol (20 mg/kg), or both together for 17 to 39 days. Noradrenaline injections increased the size of the heart, interscapular brown adipose tissue (ISBAT) and, when the injections were given at a thermoneutral environment, the metabolic activity of the ISBAT. Alprenolol injections tended to reduce the size of the heart and ISBAT and, at a thermoneutral environment, metabolic activity of the skeletal muscle. Furthermore noradrenaline treatment improved tolerance against body cooling in water at 25°C and enhanced calorogenic response to injected noradrenaline. The metabolic alterations in the rats injected with both drugs together resembled more those of the animals injected with alprenolol only than those of the noradrenaline-treated rats. It can be thus concluded that alprenolol, when given together with noradrenaline, antagonized noradrenaline-induced metabolic alterations in the organism. However the increasedpressor response to infused noradrenaline, which was evident in the alprenolol-treated animals, was not seen in the animals treated with both drugs. By contrast these animals, like also the noradrenaline-treated animals, showed decreasedpressor response to noradrenaline. This finding suggests that prolonged beta blockade does not abolish the decreased sensitivity of alpha-adrenoreceptors following prolonged noradrenaline load. On the other hand, the hypotonic response to beta-adrenergic drug, isoprenaline, was reduced by prolonged beta blockade despite simultaneous noradrenaline injections.

Key words: long-term beta blockade, enzymatic changes, brown adipose tissue, cold tolerance, non-shivering thermogenesis, adrenergic responses.

Activation of the sympathetic nervous system is known to occur in exposure and acclimation to cold. This activation can be simulated by repeated injections of noradrenaline to such an extent that noradrenaline-treated rats show a larger oxygen consumption and rectal temperature increases at cold, an increased resistance to cold, and an enlargement of brown adipose tissue (LeBlanc and Pouliot 1964, LeBlanc and Villeneuve 1970, Vallières *et al.* 1972, Hsieh and Wang 1971). This treatment, like cold acclimation, also results in enhanced sensitivity to injected catecholamines (LeBlanc and Villeneuve 1970, Vallières *et al.* 1972, Hsieh and Wang 1971), which has been interpreted to be due to increased sensitivity of adrenergic beta-receptors. Furthermore cold-acclimated and catecholamine-

tested rats have a decreased sensitivity to alpha-adrenergic drugs (Harri *et al* 1974, Tirri *et al* 1976). On the other hand, long-term pretreatment of rats with beta-blockers or chemical sympathectomy with 6-hydroxy dopamine lead to changes in metabolic and cardiovascular parameters and adrenoceptor properties (Harri 1977a, Behrens and Depois 1977, Krakoff and Ginsburg 1973), which are principally opposite to those resulting from cold-acclimation or repeated catecholamine injections (Harri and Vahola 1975).

Since beta blockers antagonize many of the acute effects of injected noradrenaline in the organism it was of interest to study whether or not prolonged beta blockade can mitigate the development of the metabolic and cardiovascular alterations following repeated noradrenaline injections. In a previous study it was shown that long-term beta blockade failed to antagonize the metabolic and adrenergic sensitivity alterations due to hyperthyroidism (Harri 1978), in spite of the fact that beta blockers have been employed with satisfactory results in the treatment of the many clinical manifestations of hyperthyroidism (Riddle and Schwarz 1978, Grossman *et al* 1971 a, b) and that beta blockers reduce the metabolic responses to exercise in hyperthyroid dogs (Kachurba, Uściłko *et al* 1976). To reduce the influence of increased release of endogenous catecholamines resulting from diminished thermoregulatory capacity due to beta-blockade (Heim and Hull 1966, Hara 1968, Irving *et al* 1974, Banet and Hensel 1977), a group of rats was also maintained at a thermoneutral temperature during the drug treatment.

Material and methods

Housing care and treatment. Male rats of Wistar/Al/Han (Han 67) strain initially weighing approximately 250 g were housed in groups of 4 and maintained on a diet of standard laboratory chow (Hankkaja, Finland) and water.

The first group, was composed of control animals, injected daily with 0.3 ml of physiological saline (group N); the second group of rats injected with 0.5 mg noradrenaline/kg (1-noradrenaline bitartrate, Fluka AG; the dose is calculated as base) (group N), the third group of rats injected with 20 mg alprenolol hydrochloride/kg (AB Hålsjö) (group A) and finally the fourth group of rats injected with 20 mg alprenolol + 0.5 mg noradrenaline/kg (group A+N). The drugs were given subcutaneously at 08.00 h five times per week for the total number of injections indicated below. These animals were kept at room temperature (19–21°C) during the drug regime. In the second series, the rats were treated with drug or control injections, but were maintained at thermoneutral temperature (33–35°C) during the injection period.

Measurements in unsedimented animals. 24 h after the 17th injection the cooling rate was measured for the rats which were swimming in water at 25°C (Dawson *et al* 1970, Harri 1978). The colonic temperature response to injected noradrenaline (0.5 mg/kg p.) was determined at thermoneutral temperature of 25°C 24 h after the 19th injection (LaBlanc and Villeneuve 1970, Harri 1978). Colonic temperatures were obtained with thermocouple inserted to depth of 4 cm, and recorded on an Ekt TE 3 (Copenhagen) potentiometer.

Cardiovascular responses. The heart rate and blood pressure responses to beta-adrenergic drug, isoproterenol, and to an alpha-adrenergic drug, noradrenaline were measured 24 h after the 31st to 39th injection. The animals were anesthetized by veterinary Nembutal (pentobarbital sodium, Abbot), 50 mg/kg injected intraperitoneally. One carotid artery was cannulated for blood pressure measurement and the opposite jugular vein for the drug infusion. The body temperature of the animals was maintained at $37 \pm 1^\circ\text{C}$ with the aid of a heat lamp. Continuous measurements of heart rate and blood pressure were made with a new gauge transducer (Hewlett Packard 267BC series), amplified with carrier amplifier (Hewlett Packard 8005B), and recorded on paper by ink-jet writing recorder (Elema, Schöander Mergograph 34).

Ex vivo assays. The same rats which were used for these studies were not subjected to the above mentioned tests. The rats were killed 24 h after the 24th injection by decapitation. Heart, adrenals and

TABLE I Effect of daily (for 24–25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on body and organ weights and on relative density of nuclei in histological sections of ISBAT. The rats were housed at room temperature (19–21°C) during the injections.

	Control (18)	Alprenolol (4)	Noradrenaline (10)	Noradrenaline + Alprenolol (10)
Δ Body weight g	102 ± 5.3	95 ± 6.4	102 ± 3.0	93 ± 3.0
Heart, mg/100 g	253 ± 4.0	234 ± 7.0	285 ± 4.8	275 ± 5.5
Adrenals, mg/100 g	21.5 ± 0.33	24.3 ± 0.84	24.5 ± 0.96	25.0 ± 1.23
ISBAT mg/100 g	113 ± 5.9	88 ± 5.7	166 ± 16.5	128 ± 6.5
Nuclei/mm in ISBAT	2 302 ± 99	3 011 ± 235	1 827 ± 151	2 029 ± 177

ISBAT = interscapular brown adipose tissue. Number of rats in parentheses. Significant difference from the controls: $p < 0.05$, $p < 0.01$ and $p < 0.001$ (Student's *t* test).

Interscapular brown adipose tissue (ISBAT) were removed and weighed. Samples of the tip of the myocardium, of the thigh muscle (*M. vastus lateralis*) and of the ISBAT were immediately frozen in liquid nitrogen, and stored at -80°C until assayed. The tissue samples were homogenized in a Potter Elvehjem glass homogenizer in Tris-HCl buffer (0.1 M, pH 7.6) to give a 2% (w/v) homogenate and centrifuged for 10 min at 1000 g at 4°C to remove broken cells and particulate debris. The supernatants were used for the determination of the activities of succinate dehydrogenase (SDH) (EC 1.3.99.1) (Earl and Konecny 1965), malate dehydrogenase (MDH) (EC 1.1.1.37) (England and Siegel 1969), citrate synthase (CS) (EC 4.1.3.7) (Sreter 1962), and hexokinase (HK) (EC 2.7.1.1) (Bass *et al.* 1969). The activity of lactate dehydrogenase (LDH) (EC 1.1.1.27) was determined using commercial reagents (Boehringer Mannheim). Measurements were made at 37°C with a Cary 118 doublebeam spectrophotometer. The assays of the four animal groups were always performed together. The protein concentration of homogenates was estimated by the phenol method with bovine serum albumin as the standard (Lowry *et al.* 1951).

For the determination of lipid content, samples of ISBAT were dried at 100°C for 2 days, and homogenized in chloroform-methanol (2:1 v/v) in turn. The total lipid content was then analyzed according to the method of Frings and Dun (1970).

Histological studies. Samples of ISBAT were fixed in 10 per cent formal. After dehydration in alcohol and embedding in paraffin, the $7\text{ }\mu\text{m}$ sections were stained with hematoxylin-eosin. The density of fat cell nuclei was calculated from photomicrographs.

Results

Body and organ weights. The results in Table I show that the weight gain was identical in all four experimental groups when the animals were housed at a room temperature during the experimental period. Noradrenaline injections increased the relative size of the myocardium. This increase was almost as pronounced in rats having received alprenolol injections in addition to noradrenaline as in the animals injected with noradrenaline only. The size of the adrenals increased in response to the drug treatments used. However the relative size of ISBAT was increased only in N-treated rats. By contrast, a significant reduction in the density of fat cell nuclei was noted in histological sections of this organ in these animals. In the A-treated group changes in the size and density of fat cell nuclei of ISBAT were the opposite to those found in N-treated group, while these parameters in A + N-rats did not differ significantly from the controls.

The results from the series of experiments carried out at $28\text{--}29^{\circ}\text{C}$ are presented in Table II. At this temperature the weight gain was significantly lower in N-treated rats than in the

TABLE II. Effect of daily (for 4-25 days) injections of alprenolol (0.2 mg/kg), noradrenaline (0.5 mg/kg) and their combination on body and organ weights and on relative density of nuclei in histological sections of ISBAT. The rats were housed at thermoneutral temperatures (28-29°C) during the injections.

	Control (10)	Alprenolol (10)	Noradrenaline (10)	Noradrenaline + Alprenolol (10)
Body weight, g	102 ± 2.1	107 ± 3.4	94 ± 2.5	97 ± 2.5
Heart, mg/100 g	299 ± 3.5	297 ± 5.3	327 ± 4.3	315 ± 3.6
Adrenals, mg/100 g	21.6 ± 1.06	22.5 ± 1.05	28.4 ± 1.24	28.0 ± 0.92
ISBAT, mg/100 g	112 ± 7.3	83 ± 3.9	118 ± 8.0	71 ± 5.2
Nuclei mm ² in ISBAT	1376 ± 123	1890 ± 156	1985 ± 199	1874 ± 74

ISBAT, interscapular brown adipose tissue. Number of rats in parentheses. Significant differences from the control: $p < 0.05$, $p < 0.01$ and $p < 0.001$ (Student *t*-test).

strols. The relative size of the heart was increased in N-treated and A + N-treated rats. The increase was, however, significantly less in the A + N group ($p < 0.05$). The enlargement of the adrenals was significantly greater in the animal groups injected with noradrenaline alone or in combination with alprenolol than in the animals having received alprenolol injections only ($p < 0.001$). The relative size of the ISBAT was smaller in the A and A + N-treated groups than in the control or N-group. The density of fat cell nuclei was, however, significantly increased in all drug-treated animal groups.

Enzymatic changes. The series of experiments performed in a conventional way at room temperature revealed no significant changes in cardiac or skeletal muscle enzyme activities (Table III).

In the ISBAT, on the other hand, the activities of oxidative enzymes, SDH, MDH and CS, tended to be low in the N-treated animals and high in the A- or A + N-treated groups. The activity of SDH was significantly higher in the alprenolol group than in the N-group ($p < 0.05$), while the activity of CS was significantly higher both in the A ($p < 0.001$) and in the A + N-group ($p < 0.001$) than in the animals having received N-injections only. Although a similar trend was observable in the activity of MDH, this trend did not attain a level of statistical significance. It is interesting to note that the activity of LDH, when expressed per unit tissue wet weight, was not decreased in the ISBAT of the N-animals, but showed an opposite trend. This is rather surprising because the protein (enzyme) concentration in the cell free tissue homogenate, which was used in the enzyme determinations, was significantly lower in this experimental group as compared to all other groups ($p < 0.01$ - $p < 0.001$). The activity of HK of ISBAT was so low that it cannot be used as an anaerobic marker enzyme for this tissue.

The decreased protein concentration of the ISBAT homogenate in N-treated animals and a similar trend in A- or A + N-treated animals is coupled with the increased amount of lipids in ISBATs of these both groups and, in addition, with the increased water content in the ISBAT of N-treated animals. It should be kept in mind that the protein concentration was determined from the 1000 \times g supernatants, not from the crude homogenate. Therefore it does not represent the total protein content of the tissue.

TABLE III Effect of daily (for 24-25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on enzyme activities and protein concentration of rat tissues and on water and lipid concentration of ISBAT. The rats were housed at room temperature (19-21°C) during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	39.1 ± 1.01	37.3 ± 1.76	38.1 ± 0.76	36.3 ± 0.69
Malate dehydrogenase	679 ± 12.5	696 ± 35.9	706 ± 15.4	658 ± 15.3
Citrate synthase	146 ± 2.37	144 ± 1.17	148 ± 3.15	141 ± 2.99
Hexokinase	3.42 ± 0.162	3.02 ± 0.240	2.99 ± 0.231	3.03 ± 0.156
Lactate dehydrogenase	941 ± 1.0	872 ± 16.1	891 ± 23.0	842 ± 17.6
Protein	88.5 ± 1.05	89.0 ± 1.25	85.0 ± 3.13	84.7 ± 1.76
M. vastus lateralis				
Succinate dehydrogenase	7.42 ± 0.263	6.92 ± 0.696	6.97 ± 0.696	7.48 ± 0.329
Malate dehydrogenase	198 ± 5.0	189 ± 13.2	187 ± 8.1	192 ± 6.1
Citrate synthase	26.2 ± 0.51	26.0 ± 1.19	24.6 ± 1.40	24.4 ± 0.93
Hexokinase	3.30 ± 0.119	3.10 ± 0.570	3.54 ± 0.155	3.53 ± 0.234
Lactate dehydrogenase	1 030 ± 24.8	1 030 ± 47.3	1 094 ± 55.7	1 057 ± 33.2
Protein	71.0 ± 1.60	68.9 ± 3.45	71.3 ± 1.55	72.0 ± 0.72
ISBAT				
Succinate dehydrogenase	23.4 ± 1.17	23.2 ± 1.76	18.4 ± 1.15	1.2 ± 1.17
Malate dehydrogenase	449 ± 24.1	473 ± 30.5	455 ± 31.5	515 ± 42.1
Citrate synthase	233 ± 24.9	281 ± 28.5	143 ± 22.4	245 ± 22.7
Hexokinase	1.39 ± 0.208	—	1.76 ± 0.234	1.22 ± 0.169
Lactate dehydrogenase	234 ± 27.2	260 ± 10.7	332 ± 47.5	269 ± 22.8
Protein	73.8 ± 3.00	79.5 ± 2.85	50.3 ± 4.55	63.9 ± 6.05
Water	34.6 ± 2.03	35.1 ± 0.85	41.7 ± 2.50	35.0 ± 1.33
Total lipids, % of dry wt.	57.3 ± 1.25	61.1 ± 2.94	63.2 ± 1.56	66.2 ± 0.68

ISBAT = interscapular brown adipose tissue. The number of rats is identical to those given in Table I. Enzyme activities are expressed as μ moles of substrate utilized per min per g wet weight, and protein as mg in cell-free tissue homogenate from 1 g of tissue. Significant difference from the controls: $p < 0.05$, $p < 0.01$ and $p < 0.001$ (Student's *t*-test).

The results in Table IV show that in the series of experiments carried out at a thermoneutral temperature the treatments used caused much more changes in the enzyme activities than was observable when the treatments were performed at a room temperature. In the myocardium, however, there were rare significant changes. The only significant differences from the control group were the decreased activity of CS in the A + N-treated group and the increased activity of MDH in the A-treated group. Furthermore, the activity of SDH was significantly ($p < 0.05$) lower in the A + N-treated group as compared to the animals having received N injections only. The protein content in the myocardial homogenate from the A-treated animal was, however, significantly ($p < 0.05$) higher as compared to N-treated or A + N-treated rats. The enzyme activities of the skeletal muscle in N-treated group were identical to those in the controls. However, the activities of oxidative enzymes, SDH, MDH and CS in the A- and A + N-groups were not only significantly lowered in comparison with the control animals but also significantly lowered in comparison with the N-group. The only exception was the difference in MDH activity between A- and N-groups.

Table IV Effect of daily (for 24-25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on enzyme activities and protein concentration of rat tissues and on water and lipid concentration of ISBAT. The rats are housed at thermoneutral temperature (25-27°C) during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	37.2 ± 1.06	38.7 ± 0.98	38.0 ± 0.84	39.3 ± 0.73
Malate dehydrogenase	748 ± 19.9	837 ± 9.9*	766 ± 12.9	742 ± 11.9
Citrate synthase	138 ± 1.67	141 ± 2.11	137 ± 2.43	131 ± 2.42
Lactate dehydrogenase	878 ± 40.8	888 ± 17.8	881 ± 32.5	913 ± 16.7
Protein	82.2 ± 2.32	84.9 ± 0.83	81.9 ± 0.98	81.9 ± 0.88
Myocardial islets				
Succinate dehydrogenase	7.63 ± 0.399	6.22 ± 0.262	7.23 ± 0.431	6.01 ± 0.250
Malate dehydrogenase	208 ± 10.4	165 ± 5.8	183 ± 8.7	153 ± 4.3*
Citrate synthase	23.2 ± 1.27	18.4 ± 0.63	20.6 ± 0.90	16.9 ± 0.40
Lactate dehydrogenase	998 ± 44.8	939 ± 22.8	943 ± 22.7	929 ± 19.3
Protein	71.4 ± 1.19	69.1 ± 1.19	67.7 ± 1.35	66.1 ± 1.19
ISBAT				
Succinate dehydrogenase	14.0 ± 1.73	26.6 ± 1.98	27.4 ± 1.58	23.7 ± 1.93
Malate dehydrogenase	238 ± 29.4	334 ± 25.9	343 ± 18.9	332 ± 13.4
Citrate synthase	93 ± 13.0	187 ± 15.0*	190 ± 11.3	154 ± 11.4
Lactate dehydrogenase	303 ± 46.1	301 ± 18.8	419 ± 29.9	331 ± 20.0
Protein	39.2 ± 1.81	74.2 ± 3.31	68.7 ± 3.46	66.8 ± 2.52
Water	—	25.0 ± 1.35	36.7 ± 1.93	34.2 ± 1.72
Total lipid, % of dry wt.	—	36.6 ± 1.27	33.7 ± 2.19	53.9 ± 2.56

ISBAT: interscapular brown adipose tissue. The number of rats is identical to those given in Table II. Enzyme activities are expressed as μ moles of substrate utilized per min per g wet weight, and protein as μ g in cell-free homogenates from 1 g of tissue. Significant differences from the controls: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

which did not attain a level of statistical significance. The activity of LDH was identical in all four experimental groups.

The metabolic activity of the ISBAT was higher in drug-treated groups than in the controls, as judged from the elevated activity of oxidative enzymes in all groups and also of the LDH in the N-treated group. Moreover the activity of CS was significantly lower in the A + N-group and that of LDH in the A- and A + N-groups in comparison to the animals having received N-injections only ($p < 0.05$). A similar trend, although statistically not significant, was observable in the activities of SDH and MDH too. The protein content of the cell free homogenate of ISBAT was higher in drug-treated rats than in the controls but no differences were found between different drug treatments. Also the fat content of the ISBAT was identical for all drug-treated groups. The water content of the ISBAT was, however, higher in the N-treated and A + N-treated rats ($p < 0.001$) than in the animals injected with alprenolol only. A comparison with Table II reveals that the density of fat cell nuclei in histological sections of ISBAT is greater in drug-treated groups. Furthermore, the fat droplets in the control ISBAT were large and mostly unilocular in comparison with the multilocular appearance in the drug-treated rats.

TABLE III Effect of daily (for 24-25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on enzyme activities and protein concentration of rat tissues and water and lipid concentration of ISBAT. The rats were housed at room temperature (18-20°C) during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	39.1 ± 1.01	37.3 ± 1.76	38.1 ± 0.76	36.3 ± 0.69
Malate dehydrogenase	679 ± 12.5	696 ± 35.9	706 ± 15.4	658 ± 15.3
Citrate synthase	146 ± 2.37	144 ± 1.17	148 ± 3.15	141 ± 2.99
Hexokinase	3.42 ± 0.162	3.02 ± 0.240	2.99 ± 0.231	3.03 ± 0.196
Lactate dehydrogenase	941 ± 21.0	872 ± 16.1	891 ± 23.0	864 ± 17.6
Protein	88.5 ± 1.05	89.0 ± 1.25	85.0 ± 1.13	84.7 ± 1.26
M. vastus lateralis				
Succinate dehydrogenase	7.42 ± 0.263	6.92 ± 0.696	6.97 ± 0.696	7.49 ± 0.339
Malate dehydrogenase	198 ± 5.0	189 ± 13.2	187 ± 8.1	184 ± 6.1
Citrate synthase	26.2 ± 0.51	26.0 ± 1.19	24.6 ± 1.40	24.4 ± 0.93
Hexokinase	3.30 ± 0.119	3.10 ± 0.570	3.54 ± 0.155	3.53 ± 0.284
Lactate dehydrogenase	1030 ± 4.8	1030 ± 47.3	1094 ± 55.7	1057 ± 33.2
Protein	71.0 ± 1.60	68.9 ± 3.45	71.3 ± 1.55	72.0 ± 0.71
ISBAT				
Succinate dehydrogenase	23.4 ± 1.17	23.2 ± 1.76	18.4 ± 1.15	14 ± 1.17
Malate dehydrogenase	449 ± 24.1	473 ± 30.5	435 ± 31.3	515 ± 42.1
Citrate synthase	233 ± 4.9	281 ± 28.5	143 ± 22.4	45 ± 22.7
Hexokinase	1.39 ± 0.208	—	1.76 ± 0.234	1.22 ± 0.169
Lactate dehydrogenase	254 ± 27.2	260 ± 10.7	332 ± 47.5	269 ± 22.8
Protein	73.8 ± 3.00	79.5 ± 3.85	50.3 ± 4.55	63.9 ± 6.05
Water	34.6 ± 2.03	35.1 ± 0.85	41.7 ± 1.50	33.0 ± 1.31
Total lipids, % of dry wt.	57.3 ± 1.25	61.1 ± 2.94	63.2 ± 1.56	66.2 ± 0.66

ISBAT = Interacapsular brown adipose tissue. The number of rats is identical to those given in Table I. Enzyme activities are expressed as μ moles of substrate utilized per min per g of wet weight, and protein as mg in cell-free tissue homogenates from 1 g of tissue. Significant difference from the controls: $p < 0.01$ and $p < 0.001$ (Student's *t*-test).

The results in Table IV show that in the series of experiments carried out at a thermoneutral temperature the treatments used caused much more changes in the enzyme activities than was observable when the treatments were performed at a room temperature. In the myocardium, however, there was rare significant changes. The only significant differences from the control group were the decreased activity of CS in the A + N treated group and the increased activity of MDH in the A treated group. Furthermore, the activity of SDH was significantly ($p < 0.05$) lower in the A + N-treated group as compared to the animals having received N injections only. The protein content in the myocardial homogenate from the A-treated animal was, however, significantly ($p < 0.05$) higher as compared to N treated or A + N treated rats. The enzyme activities of the skeletal muscle in N-treated group were identical to those in the controls. However, the activities of oxidative enzymes, SDH, MDH and CS, in the A and A + N-groups were not only significantly lowered in comparison with the control animals but also significantly lowered in comparison with the N-group. The only exception was the difference in MDH activity between the A and N-groups.

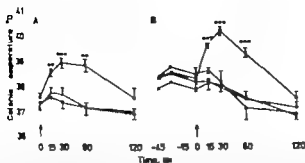


Fig. 2. The response of the colonic temperature to injected noradrenaline (0.5 mg/kg *p*) at 28°C in control rats (Δ), in alprenolol-treated rats (20 mg/kg daily for 19 days) (●), in noradrenaline-treated rats (0.5 mg/kg daily for 19 days) (○), and in rats injected with alprenolol together with noradrenaline (x). All the animals were maintained at 28–29°C during the injection periods and all the animals were kept at room temperature. Vertical bars indicate \pm S.E. Noradrenaline was injected at 0 min. Asterisks mark those hyperthermic changes which significantly differ from the control after (10 min). * $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test). 4 rats per group.

the pre-load treatment and in the animals maintained at a thermoneutral temperature, pre-treatment resulted in enhanced sensitivity to noradrenaline. This was seen by raised colonic temperature after the test injection. In other experimental groups noradrenaline test injection failed to cause hyperthermia.

Fig. 3 shows the heart rate and blood pressure responses to infused noradrenaline and isoprenaline in anesthetized rats. This test was performed only on the rats, which were maintained at room temperature. The basic heart rates for the control, A, N- and A+N-treated groups were 435 ± 6.8 , 407 ± 4.8 , 409 ± 7.8 and 406 ± 8.9 beats/min, respectively. Thus the heart rates in all drug-treated groups were significantly slower in comparison with the controls ($p = 0.02$ to $p = 0.01$). The initial systolic blood pressure values varied from the average value of 145 ± 4.8 mmHg in the controls to 159 ± 8.9 mmHg in the N-treated group, and the diastolic from 118 ± 3.8 to 124 ± 6.8 mmHg, respectively. No significant differences between various groups were found. The slight bradycardia caused by noradrenaline infusion was identical in all groups, while the tachycardic response to infused isoprenaline was significantly greater in the A- and N-treated animals than in the control or A+N-treated group. The pressor response to infused noradrenaline was significantly smaller in the N- and A+N-groups, while the rise of the diastolic blood pressure caused by noradrenaline was significantly greater in the A-treated animals. On the other hand, the hypotonic response to infused isoprenaline was identical in N-treated group as in the controls but significantly smaller in the A- and A+N-treated animals.

Discussion

In accordance with the findings of LeBlanc and Villeneuve (1970) repeated injections of noradrenaline induced marked cardiomegaly in the rats. This effect was as marked in animals maintained at a room temperature during the treatment as in the animals maintained at a thermoneutral temperature. It was only partially antagonized by simultaneous

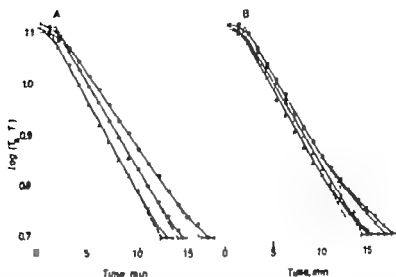


Fig. 1 The rate of body cooling in rats swimming in water at 25°C. Δ —control rats, \bullet —alprenolol treated rats (20 mg/kg daily for 17 days), \circ —noradrenaline treated rats (0.5 mg/kg daily for 17 days) and \times —rats injected with alprenolol together with noradrenaline. A the animals were maintained at 28–29°C during the injection periods and B the animals were kept at a room temperature (19–21°C). $T_b - T_a$ —the difference of body and ambient (water) temperature. The time of the body temperature to cool to 30°C is indicated at the end of each line as the mean \pm S.E. The broken lines extrapolate the initial rates of body cooling. 5 rats per group.

Whole animal experiments Fig. 1 shows the rate of cooling of rats swimming in water at 25°C. In the series of experiments constructed at a room temperature the initial rate of body cooling was almost identical in all four experimental groups. The cooling constants for the initial phase of body temperature drop were calculated according to the formula of $C = -2.3 d \log (T_b - T_a) / dt$ (Morrison and Tietz 1957) where T_b and T_a refer to body and ambient temperature respectively. They were 4.33 ± 0.10 , 4.22 ± 0.16 , 4.21 ± 0.17 and 4.48 ± 0.14 C/h for the control, N, A and A+N-groups, respectively. These values do not differ significantly from each other. However, when the differences between ambient and body temperature becomes smaller in a living animal, the metabolic heat production slows down the rate of body cooling. This can be seen by a shift of the cooling rate curve to the right from the initial slope. Owing to this shift, the time of the body temperature to cool to 30°C was significantly longer for the N or A treated groups than for the controls or A+N-treated group ($p < 0.05$ to $p < 0.01$) being 17.9 ± 0.57 , 17.0 ± 0.54 , 15.5 ± 0.62 and 15.1 ± 0.71 min for the N, A, control and A+N groups, respectively.

On the other hand, in the series of experiments in which the animals were kept at 28–29°C during the drug injections, there was much marked differences between the various drug treated groups. The cooling constants, which were 4.89 ± 0.09 , 4.52 ± 0.18 and 3.91 ± 0.10 C/h for the A+N, A and N-treated groups, respectively, did differ significantly from each other ($p < 0.05$ – $p < 0.01$). Furthermore, the times of the body temperature to cool to 30°C were 12.8 ± 0.65 , 14.5 ± 0.41 and 17.0 ± 0.68 min for the A+N, A and N-treated groups, respectively; they did differ significantly from each other ($p < 0.05$ to $p < 0.01$).

Fig. 2 shows the metabolic response of the rats to injected noradrenaline (0.5 mg/kg i.p.) when measured at 28°C. Both in the animals maintained at a room temperature during

supported also by the results obtained from the series of experiments performed at a room temperature. At this environment the impaired thermoregulatory capacity due to beta blockade, results in a compensatory release of endogenous noradrenaline (cf. Harri 1971a, b, Irving *et al.* 1974), which more or less compensates for the effectiveness of beta blockade. At a thermoneutral environment there is no need for extra heat production and, as a result, for extra release of catecholamines. It is interesting to note, however, that even at the higher temperature repeated noradrenaline injections failed to prevent the influence of alprenolol. This may be due to the shorter duration of the action of noradrenaline (cf. Fig. 4, Harri 1977b) as compared with that of alprenolol. These results also emphasize that to perform animal experiments in a conventional way at a room temperature is not always the proper way.

The activation of the brown fat in a cold environment is mainly due to the direct effect of the sympathetic nervous system on this tissue (Seydoux 1977). Thus repeated injections of noradrenaline in order to mimic the enhanced release of this amine which occurs at cold should lead to changes, similar to those resulting from cold acclimation. The elevated size of this tissue, as observed in this study for 19–21 °C-acclimated rats and earlier by LeBlanc and Villeneuve (1970), demonstrate that this is the case. Furthermore, this enlargement is partially antagonized by simultaneous alprenolol treatment.

Unlike at room temperature, N-treatment did not affect the absolute or relative size of the ISBAT at 28–29 °C. The metabolic activity of the tissue was, however, greatly increased by N-treatment, as judged from the enzymatic changes and multilocular picture of the tissue. However, it is difficult to state whether or not alprenolol antagonized the effect of noradrenaline, because A-treatment alone induced very similar enzymatic changes. At this higher temperature A-treatment alone reduced the size of ISBAT. This effect is unaffected despite simultaneous N-treatment. It is thus tempting to speculate that the influence of alprenolol was stronger than that of noradrenaline when these drugs were given simultaneously.

In the cooling rate test the antagonizing effect of alprenolol against noradrenaline induced improvement in the ability of rats to withstand body cooling in water was very clear. In fact, the rate of body cooling was even more rapid in the rats having received alprenolol injections in addition to noradrenaline than in the rats having received alprenolol systems only. In swimming rats the rate of body cooling depends on the insulative capacity of the body and on the heat production capacity. When the rats are forced to move, this increases the rate of heat loss by convection, and the insulative properties of the body are of less importance. In swimming rats the rate of body temperature loss is even lower than that of a dead body which has no heat production (Dawson *et al.* 1970). Thus it can be speculated that in swimming rats the rate of body cooling is mainly dependent on the heat production capacity of the body and the possible differences in the cooling rate do not reflect differences in the heat production capacity. Based on this test, it can be concluded that alprenolol injections very clearly abolished the effect of N-treatment on the heat production capacity.

Increased non-shivering thermogenesis, i.e. calorogenic response to injected noradrenaline has been reported to result from repeated noradrenaline treatment (LeBlanc and Pouliot

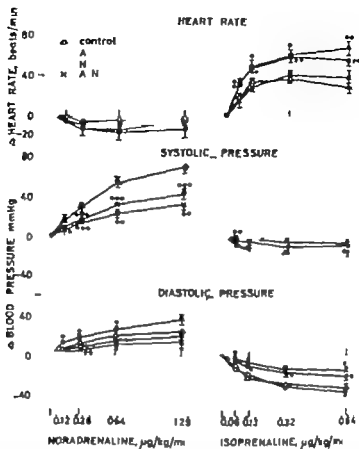


Fig. 3 Effect of noradrenaline and isoprenaline infusions on heart rate, and systolic and diastolic blood pressure in anesthetized rats. A - alprenolol treated rats (20 mg/kg daily for 31-39 days), N - noradrenaline treated rats (0.5 mg/kg daily for 31-39 days) and A + N rats injected with alprenolol together with noradrenaline. Each point represents the mean \pm S.E. of 8 to 10 animals. Responses that differ significantly from controls are marked by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

alprenolol injections in spite of the fact that alprenolol injections alone did not affect the heart size.

Despite marked cardiomegaly there was, however, no or only minor enzymatic changes in the myocardium of these animal groups. It is interesting to note that physical training, although it also results in marked cardiomegaly or metabolic changes in the skeletal muscle, induces only minor changes in the myocardial enzymes (Gollnick and Januzzo 1972, Baldwin *et al.* 1977, Harri 1977 b).

In the skeletal muscle enzymatic changes were observable only in the series of experiments which were performed at a thermoneutral temperature. In these experiments, a decrease in the activity of the aerobic enzymes was evident both in animals injected with alprenolol only and in the rats having received alprenolol injections in addition to noradrenaline. Since a similar decrease was not observed for animals treated with N only and since it has been shown that prolonged stimulation of the organism by a beta adrenergic agonist, isoprenaline (Harri and Valtola 1975) can increase the oxidative metabolism in the skeletal muscle, it is tempting to conclude that this decrease results from diminished sympathetic influence on the beta adrenoceptors due to prolonged beta blockade. The

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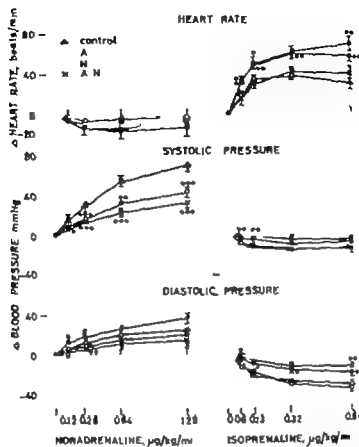


Fig. 3 Effect of noradrenaline and isoprenaline infusions on heart rate, and systolic and diastolic blood pressure in anesthetized rats. A = alprenolol-treated rats (20 mg/kg daily for 31–39 day), N = noradrenaline-treated rats (0.5 mg/kg daily for 31–39 day) and A + N = rats injected with alprenolol together with noradrenaline. Each of 6 represents the mean \pm S.E. of 8 to 10 animals. Responses that differ significantly from controls are marked by asterisks: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's *t*-test).

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Despite marked cardiomegaly there was, however, no or only minor enzymatic changes in the myocardium of these animal groups. It is interesting to note that physical training, although it also results in marked cardiomegaly or metabolic changes in the skeletal muscle, induces only minor changes in the myocardial enzymes (Gollnick and Januzzo 1972, Baldwin *et al.* 1977, Harri 1977b).

In the skeletal muscle, enzymatic changes were observable only in the series of experiments which were performed at a thermoneutral temperature. In these experiments, a decrease in the activity of the aerobic enzymes was evident both in animals injected with alprenolol only and in the rats having received alprenolol injections in addition to noradrenaline. Since a similar decrease was not observed for animals treated with N only and since it has been shown that prolonged stimulation of the organism by a beta adrenergic agonist, isoprenaline (Harri and Valtola 1975), can increase the oxidative metabolism in the skeletal muscle, it is tempting to conclude that this decrease results from diminished sympathetic influence on the beta adrenoceptors due to prolonged beta blockade. This

is supported also by the results obtained from the series of experiments performed at a room temperature. At this environment the impaired thermoregulatory capacity due to beta blockade, results in a compensatory release of endogenous noradrenaline (*cf* Harri 1977a, b, Irving *et al.* 1974), which more or less compensates for the effectiveness of beta blockade. At a thermoneutral environment there is no need for extra heat production and, as a result, for extra release of catecholamines. It is interesting to note, however that even at this higher temperature repeated noradrenaline injections failed to prevent the influence of alprenolol. This may be due to the shorter duration of the action of noradrenaline (*cf* Fig. 2, Harri 1977 b) as compared with that of alprenolol. These results also emphasize that to perform animal experiments in a conventional way at a room temperature is not always the proper way.

The activation of the brown fat in a cold environment is mainly due to the direct effect of the sympathetic nervous system on this tissue (Seydoux 1977). Thus repeated injections of noradrenaline in order to mimic the enhanced release of this amine which occurs at cold should lead to changes, similar to those resulting from cold acclimation. The elevated size of this tissue, as observed in this study for 19–21°C-acclimated rats and earlier by LeBlanc and Villeneuve (1970), demonstrate that this is the case. Furthermore, this enlargement is partially antagonized by simultaneous alprenolol treatment.

Unlike at room temperature, N-treatment did not affect the absolute or relative size of the ISBAT at 28–29°C. The metabolic activity of the tissue was, however greatly increased by N-treatment, as judged from the enzymatic changes and multilocular picture of the tissue. However it is difficult to state whether or not alprenolol antagonized the effect of noradrenaline, because A-treatment alone induced very similar enzymatic changes. At this higher temperature A-treatment alone reduced the size of ISBAT. This effect was unaffected despite simultaneous N-treatment. It is thus tempting to speculate that the influence of alprenolol was stronger than that of noradrenaline when these drugs were given simultaneously.

In the cooling rate test the antagonizing effect of alprenolol against noradrenaline induced improvement in the ability of rats to withstand body cooling in water was very clear. In fact, the rate of body cooling was even more rapid in the rats having received alprenolol injections in addition to noradrenaline than in the rats having received alprenolol injections only. In swimming rats the rate of body cooling depends on the insulative capacity of the body and on the heat production capacity. When the rats are forced to swim, this increases the rate of heat loss by convection, and the insulative properties of the body are of less importance. In swimming rats the rate of body temperature loss is even faster than that of a dead body which has no heat production (Dawson *et al.* 1970). Thus it can be speculated that in swimming rats the rate of body cooling is mainly dependent on the heat production capacity of the body and the possible differences in the cooling rate also reflect differences in the heat production capacity. Based on this test, it can be concluded that alprenolol injections very clearly abolished the effect of N-treatment on the heat production capacity.

Increased non-shivering thermogenesis, *i.e.* calorogenic response to injected noradrenaline has been reported to result from repeated noradrenaline treatment (LeBlanc and Pouliot

1964 LeBlanc and Villenave 1970 Hsieh and Wang 1971). In this study the hypertensive response to injected noradrenaline was used as a measure of non-shivering thermogenesis. The results showed that repeated N-injections increased this response both in the rats maintained at a room temperature and in the rats treated at a thermoneutral temperature. This increased response was very clearly abolished by simultaneous A injections.

After repeated N injections the rats showed decreased hypertensive response to intravenously infused noradrenaline, while in the rats having received repeated alprenolol injections noradrenaline caused a greater increment in the diastolic pressure. In this case however the A + N-rats very closely resembled those of N-treated rats, an indication that A treatment did not abolish the changes following N-treatment. Since the pressor response to noradrenaline is mainly due to vasoconstriction, which is known to be mediated by the alpha-adrenoreceptors, it was to be expected that beta blockade had no antagonistic effect on these changes.

By contrast, isoprenaline is known as a pure beta agonist. Therefore, it could be expected that, if prolonged noradrenaline treatment could result in changes in responsiveness to isoprenaline, these changes should be antagonized by alprenolol injections. The present results show however that repeated N injections resulted in enhanced tachycardic response to infused isoprenaline which was also observable in the A treated rats but not in the A + N treated rats. An increased tachycardic response to infused isoprenaline has been reported to occur after repeated noradrenaline injections (Vallieres *et al.* 1972). Since similar sensitization also results from repeated injections of isoprenaline (LeBlanc *et al.* 1972, Vallieres *et al.* 1972) it has been concluded that this kind of supersensitivity results from prolonged stimulation of beta receptors. The supersensitivity following prolonged A treatment is more difficult to explain. A possible explanation is the intrinsic sympathomimetic activity of alprenolol, which in repeated use could activate the cardiac beta receptors. In this case the supersensitivity should be observable in the A + N-treated animals as well. Since this was not the case this problem remains to be clarified by further experiments.

The unchanged response of the blood pressure to isoprenaline in the N-treated rats is not in accordance with the findings of Vallieres *et al.* (1972) they found a decreased hypotonic response to isoprenaline in these animals. In general, the hypotonic and tachycardic responses to isoprenaline were much more greater in their studies than those observed in the present study with comparable doses of the drug and with a comparable experimental technique. Thus genetic differences between their rats and the animals used in this study are possible. As to the changed response to isoprenaline in the present study it seems obvious that A-treatment both alone and in combination with noradrenaline led to a decreased hypotonic response to isoprenaline. If it is assumed that this response results from isoprenaline-induced vasodilatation, which is mediated by the beta₂-receptors (Lands *et al.* 1967), unlike beta₁-receptor mediated cardiac responses, it can be concluded that prolonged beta blockade leads to subsensitivity of beta₂-receptors. This effect persists despite simultaneous stimulation of those receptors by noradrenaline, an indication that in this case, too, the influence of beta blocking effect of alprenolol exceeds that of beta stimulating effect of noradrenaline, when these both drugs are given together.

However, it should be kept in mind that reflex adjustment of circulation greatly affects the cardiovascular reactions in the whole animal experiments. Therefore, altered responses to sympathomimetic amines are not necessarily explained by sensitivity changes of adrenergic receptors. In vivo-experiments are needed to clarify these problems in more details.

As explainable phenomenon is the enlargement of the adrenals in the 28-79 C acclimated animals, because it was as remarkable in the A + N-group as in the N-treated group. If this enlargement can be interpreted to be due to the stress of repeated noradrenaline loads, it should be absent in the A + N-group, in which, according to the other data presented, alprenolol abolished the effect of noradrenaline. Since this was not the case, this phenomenon cannot be accounted for by the alprenolol-noradrenaline antagonism principle.

In conclusion, the present results show that, if repeated noradrenaline injections resulted in metabolic changes, these changes were abolished by simultaneous alprenolol injections. In those cases in which noradrenaline injections did not induce metabolic alterations but alprenolol injections did, the metabolic alterations in the animals having received both drugs simultaneously more closely resembled those of the animals injected with alprenolol only. The cardiovascular sensitivity changes resulting from N-treatment, which can be expected to be mediated by the alpha-adrenoreceptors, were not antagonized by alprenolol treatment, while at least some of the changes mediated by the beta-adrenoreceptors, were. These results demonstrate that prolonged alprenolol treatment can protect the organism against the metabolic changes resulting from repeated noradrenaline loads associated with stress situations.

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Inhibition of compound 48/80-induced intradental sensory nerve activity by disodium cromoglycate and serotonin antagonists

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Abstract

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Local application of compound 48/80 induced long lasting nerve activity in intradental sensory nerves in the teeth of cats and dogs. This effect was inhibited by pretreatment with disodium cromoglycate (DSCG) given locally (10^{-4} M) or i.a. (20 mg/mm). DSCG did not have any effect on the nerve excitability *per se* as judged from the responses to hypertonic NaCl and air blasts applied to exposed dentin before and after administration of DSCG. Local treatment with tyramine acid diethylamide (0.1-1 mg/ml) and methysergide (0.05-0.3 mg/ml) selectively reduced or inhibited the compound 48/80 induced nerve activity. Systemic administration of methysergide (12.5 µg/kg) prevented the excitatory effects of compound 48/80 but was without effect when administered during state of established activity. The present findings support the hypothesis that compound 48/80 has an indirect effect on intradental sensory nerves and indicate that vascular reactions take part in intradental sensory nerve excitation.

It is as recently demonstrated that compound 48/80 causes increased excitability in the sensory nerves in the feline tooth after local application of the drug to exposed dentin (Olqvist 1974). These results raised the question of whether this effect of compound 48/80 was due to a direct action on the nerve fibres or an indirect effect mediated by biogenic substances released from mast cells. The latter alternative implies that the induced nerve activity may be a sign of an acute inflammatory reaction in the pulp. However little is known about the contribution of such reactions to sensory nerve activation and pain in the tooth. Therefore, in the present investigation we have analyzed by pharmacological means the excitatory action of compound 48/80 in teeth of cats and dogs.

The present results strengthen the hypothesis that compound 48/80 induces vascular changes in the pulp and suggest that 5-HT is involved in the resulting sensory nerve activity.

Methods

Adult cats, anesthetized with chloralose-urethane and female dogs anesthetized with pentobarbital were used for the experiments. The tooth was subtotomized and provision made for recording the femoral blood pressure. The experimental set up is similar to that described by Olqvist *et al.* (1974).

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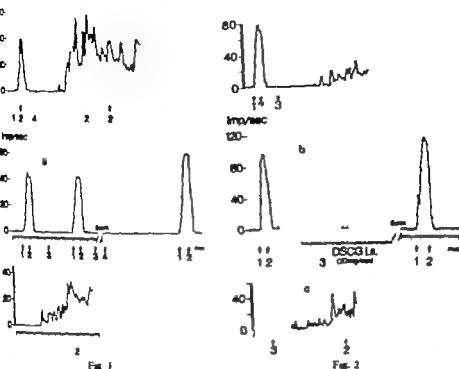


Fig. 1. Influence of compound 48/80 on sensory nerve activity in the dental pulp. a. expected response to locally applied drugs in the control tooth. b. similar procedures in contralateral tooth after local pretreatment with desodine chromoglycate (DSCG). same tooth as in Fig. 1b. after treatment with DSCG. 1. Sodium chloride, 1.54 M. 2. Sodium chloride, isotonic. 3. DSCG, 2 $\times 10^{-4}$ M. 4. Compound 48/80, 1 mg/ml. Impulse frequency represents the running average for 10 sec interval and is given as imp/sec. Call, calibration.

Fig. 2. The effect of local application of compound 48/80 on intradental sensory nerve activity before and after intradermally administered desodine chromoglycate (DSCG). a. responses in control tooth prior to DSCG administration. b. contralateral tooth during infusion of DSCG (20 mg/min). Note that stimulation with hypertonic sodium chloride induced similar responses before and after DSCG infusion. same tooth as in Fig. 1b. later. The following solutions were applied locally: 1. Sodium chloride, 0.76 M. 2. Sodium chloride, isotonic. 3. Compound 48/80, 0.1 mg/ml.

Serotonin antagonists

The principal action of DSCG is to stabilize the mast cell membrane (Brodeur *et al.* 1974). The ability of this drug to prevent the excitation induced by compound 48/80, but not the effect of other well-known direct acting stimuli, suggests that compound 48/80 has an indirect effect on the sensory fibres, e.g. by releasing some biogenic principle. Since serotonin (5-HT) has been shown to excite intradental neurones (Olgart 1974) application of serotonin antagonists could give further information as to the mechanism of excitation by compound 48/80. Fig. 3 shows the results from one experiment which demonstrates the effect of locally applied LSD on the compound 48/80 induced activity. The compound was washed out from the cavity and replaced by isotonic saline 10 min before LSD was introduced. As can be seen, LSD (0.1 mg/ml) (Fig. 3) caused a partial inhibition of the activity within 30 s.

Two platinum electrodes were inserted into dentinal cavities prepared in the canine tooth. The cavities were filled with isotonic saline and differential recordings were made between the two electrodes. Signals were displayed on a cathode ray tube and fed into equipment for frequency analysis (Edwall and Scott 1971, Haegerstrom 1976 a). The potentials obtained using this method have earlier been shown to originate from intradental sensory fibres (Arwill *et al* 1973) and to be associated with pain in man (Edwall and Olgart 1977). As has been previously demonstrated (Haegerstrom 1976 c) the present technique enables recordings to be made from a small number of units (generally less than five). No attempts were made in this study to differentiate between impulses of different amplitude. The effects of pharmacological agents are quantitatively studied by measurements of changes in total impulse activity. For further details concerning the recording technique and analysis see Haegerstrom (1976 c).

Compound 48/80 solution (0.1 μ l) was introduced into the incisal cavity after removal of the enamel solution. Disodium cromoglycate (DSCG) was administered either by application into the incisal cavity or by intraarterial infusion in the posterior auricular artery close to its junction with the external maxillary artery. Lysergic acid diethylamide (LSD) and methysergide were administered either locally into the incisal cavity or by infusion. Sodium chloride solution (0.76 M-1.54 M) was used locally in the cavity. The latent period of the resulting nerve response is dependent on cavity depth (Olgart 1974) and is used to standardize the preparations when results in pairs of teeth were to be compared. The maximal frequency response to this procedure was also used to test the excitability of the sensory neurons after the various procedures. Aconitine was applied locally into the incisal cavity in some experiments to produce long lasting nerve activity (Haegerstrom 1976 b) which served as control. The drugs are dissolved in isotonic saline.

Results

Disodium cromoglycate

Pairs of lower canine teeth (4 cats and 2 dogs) were used in this series of experiments. In each animal one of the teeth served as control to demonstrate the expected response to hypertonic NaCl and compound 48/80. In the contralateral tooth the cavity was pretreated with DSCG solution (2×10^{-3} M) for 5 min, which was then replaced with isotonic NaCl. Hypertonic NaCl was then applied followed by compound 48/80 solution. It was a consistent finding that DSCG prevented the nervous response to compound 48/80 (1 mg/ml) but not that to 1.54 M NaCl. Fig. 1 shows the result obtained in such an experiment. As can be seen, a response to compound 48/80 was obtained 30 min later in the tooth previously treated with DSCG. This recovery in response was a consistent finding in all experiments. In 4 of the experiments air blasts were included. This stimulus was applied to the exposed dentin in the recording cavity before and after application of DSCG. Comparison between the two responses showed that the excitability was not influenced by DSCG treatment. When DSCG was given after compound 48/80 (5 procedures in 3 cats) no inhibition or a weak inhibitory effect on the activity was observed.

In a similar series of experiments (3 cats) DSCG was administered by intra-arterial infusion (20 mg/min) before and during the local application of compound 48/80 (100 μ g/ml). The results were essentially the same as those reported above. Compound 48/80 failed to induce any nerve activity during the DSCG infusion (9 min) and during the subsequent 10 min (Fig. 2). However the response to 0.76 M NaCl or to air blasts (not shown in the figure) was unchanged when compared with the controls made before the DSCG treatment. When compound 48/80 was applied 90 min after the previous DSCG infusion, it induced nervous activity (Fig. 2 c-3) of similar magnitude to that in the contralateral control tooth (Fig. 2 a-3).

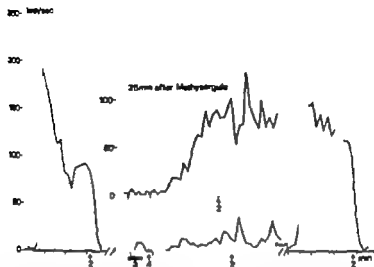


Fig. 1. The effect of compound 48/80 on intradental sensory nerve activity after intravenous administration of methysergide, 12.5 μ g/kg (3). The following solutions are applied locally in the recording cavity: 1. Sodium chloride, 0.76 M. 2. Sodium chloride, isotonic. 3. Compound 48/80, 1 mg/ml. 4. Compound 48/80, 1 mg/ml. 1 sec shows the response in the same tooth 25 min later.

In 4 expts. methysergide (12.5 μ g/kg) was administered i.v. 1 min before the application of compound 48/80. A typical result is shown in Fig. 5. As can be seen, application of compound 48/80 (Fig. 5, 4) after the methysergide administration (Fig. 5, 3) induced activity of low frequency during the following 15 min. The response to 0.76 M NaCl obtained thereafter was similar to the initial control response (Fig. 5, 1). Twentyfive min later a new application of compound 48/80 induced a higher frequency response, which was similar to that obtained in the contralateral control tooth before methysergide administration (not shown in the figure). In contrast to these results, it was found in 3 expts. that methysergide given 1 min after compound 48/80 was unable to inhibit the activity already induced. In 5 control expts. in which aconitine was used to excite the neurons the resulting activity could neither be prevented by a previous injection of methysergide nor be influenced by an injection of methysergide during the established activity.

Tetrodotoxin

The finding that methysergide did not have any effect on the nerve activity when given after compound 48/80 may be explained by a local circulatory arrest in the pulp induced by compound 48/80. An effective distribution of the blocking agent to the pulp would then be prevented. This hypothesis was tested in 3 additional expts. in the following way: Tetrodotoxin (TTX, 3 μ g/kg) was given i.v. when intradental nerve activity had been induced by compound 48/80 (0.5 mg/ml) and aconitine (10^{-6} g/ml) in two separate teeth in the same animal. Aconitine is known to act directly on nerves by depolarization of the nerve endings (Kilbinger 1968). TTX, has earlier been shown to effectively block aconitine-induced nerve activity as well as activity induced by other means in this preparation (Hägerström 1976 c).

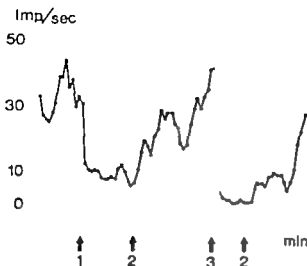


Fig. 3. The effect of lysergic acid diethylamide (LSD) on the sensory unit impulse frequency elicited by compound 48/80 (0.1 mg/ml). Local application in the recording cavity. 1, 3 LSD 0.1 and 1 mg/ml respectively. 2. Sodium chloride isotonic.

After washing the cavity with isotonic saline, the activity gradually increased during the following 3 min. LSD (1 mg/ml) (Fig. 3, 3) caused an almost total inhibition of the activity. Similar results were obtained with methysergide. In Fig. 4, 1 it can be seen that methysergide (0.5 mg/ml) caused a total inhibition of the compound 48/80-induced activity within 1 min after its application. Replacement of the drug by hypertonic NaCl solution (0.76 M) together with methysergide at the same concentration (Fig. 4, 2) induced a rapid increase in activity. This response indicated that the blocking effect of methysergide of compound 48/80 induced activity was not due to local anesthetic effect. After washing the cavity with isotonic saline (Fig. 4, 3) the activity was gradually restored to the initial level. The results in Fig. 4, 4 and 4, 5 show that two applications of methysergide at lower concentrations (0.1 mg/ml, 0.05 mg/ml) markedly reduced the activity. These results represent the typical response obtained in 15 procedures in 12 cats. When LSD and methysergide were used in the same preparation, methysergide was generally found to be the more potent in inhibiting the compound 48/80 induced activity.

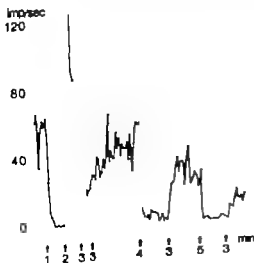


Fig. 4. The effect of methysergide on the sensory unit impulse frequency elicited by compound 48/80. Local application in the recording cavity. 1, 4, 5 Methysergide 0.5, 0.1 and 0.05 mg/ml respectively. 2. Methysergide 0.5 mg/ml in a solution of sodium chloride, 1.54 M. 3. Sodium chloride, isotonic. Note the response to hypertonic sodium chloride with methysergide.

In conclusion, the present results strengthen the hypothesis that compound 43/80 activates intradental sensory nerves by an indirect mechanism. It is suggested that compound 43/80 induces scalar changes in the pulp possibly involving 5-HT.

The study was supported by grants from the Swedish Medical Research Council (B76-24X-816-11) and Lundberg's Institute.

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In the present experiments, the activity in the aconitine treated tooth was rapidly abolished, while in the compound 48/80 treated tooth the activity was unaffected by the systemically administered dose of TTX. In contrast to these results, local applications of TTX (10^{-4} g/ml) in the teeth were shown to rapidly abolish both types of induced activity. These findings demonstrate that the same local concentration of TTX can block the nerve activity induced by both substances and suggest that compound 48/80 may cause some change in the local circulation under the cavity resulting in an incomplete distribution of the systemically administered TTX to the activated nerves.

Discussion

The inhibition of compound 48/80-induced nerve activity by DSCG in the present expts. is interesting since the drug has a stabilizing effect on the mast cell membrane, but few other pharmacological effects (Cox 1971 Brodgen *et al* 1974 Nuki and Farnoush 1975). The mast cell degranulation induced by compound 48/80 in rat peritoneal tissue was reduced by local pretreatment with DSCG in concentrations similar to those used in the present study (Orr *et al* 1971 Marshall 1972). In the present expts, a direct action of DSCG on the sensory nerve excitability may be ruled out, since the preparation showed normal responses to application of hypertonic NaCl and air blasts after DSCG treatment. Our results thus strengthen the hypothesis that nerve excitation induced by compound 48/80 involves an indirect mechanism in which mast cells play a part.

The question arises whether principles released from mast cells have a direct action on the nerves or if the nerve activity is due to some indirect mechanism involving these substances. A direct effect seems less probable since it has been shown that substances which are released or formed as a consequence of cat mast cell degranulation are without excitatory effect in the present preparation (Olgart 1974 Haeagerstam and Edwall 1975 Gazelius *et al* unpublished).

The other alternative is that circulatory changes may be involved. Support for this assumption is found in recent results from studies on the cat tooth (Ahlberg and Edwall 1977 Ahlberg 1978) showing that local application of compound 48/80 reduces pulp blood flow concomitantly with an increase in sensory nerve activity. Further support for a vascular influence is to be found in the results of the present experiments where systemically administered TTX was unable to block nerve activity induced by compound 48/80 but blocked that induced by aconitine. This suggests that compound 48/80 or substances released from mast cells, induces vascular changes that initiate the neural response. Such a mechanism of excitation may be related to a local increase in intrapulpal pressure due to outward filtration of fluid from the vessels (van Hassel 1971) and pressure effects on mechanosensitive sensory receptors (Paintal 1976). A mechanosensitive feature of intradental sensory neurons was suggested by Haeagerstam (1976 c).

The present finding that methysergide in a dose range of 10–15 μ g/kg was able to block nerve activity induced by compound 48/80 may indicate that 5-HT is involved. 5-HT may take part in the nerve excitation either by exerting direct effect on the nerves (Olgart 1974) or indirectly by its vascular actions.

conclusion, the present results strengthen the hypothesis that compound 48/80 activates dental sensory nerves by an indirect mechanism. It is suggested that compound 48/80 induces vascular changes in the pulp possibly involving 5-HT.

This study was supported by grants from the Swedish Medical Research Council (B76-24X-816-11) and Karolinska Institute.

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Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland

By

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Abstract

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Lactose secretion by goat mammary glands was studied after intra-arterial infusions of insulin and glucose causing rises in plasma insulin concentrations in mammary venous blood of about 3-5 mg/ml. Such levels are sufficient to strongly stimulate insulin sensitive processes when present in the systemic circulation. Two goats with mammary glands *in situ* and two with one of the glands autotransplanted to the neck were used for the experiments. Increasing the insulin concentration in blood to the mammary gland induced a decrease in lactose yields during 3 h infusion ($p < 0.02$). Simultaneously arterial plasma glucose decreased by 15-20 mg/100 ml to about 50 mg/100 ml. When the systemic hypoglycemic effect of the insulin infusion was offset by a simultaneous intra-venous infusion of glucose no significant change in lactose secretion was observed during 8 hours of hyperinsulinemia. No change in mammary glucose uptake as measured by mammary blood flow-mammary (arterio-venous) glucose differences could be detected. (Average glucose uptake for 2 h before infusion, 30.9 mg/min; during 8 h of infusion, 31.2 mg/min.) It may be concluded that increased levels of insulin in the blood perfusing the mammary gland did not affect mammary glucose uptake or lactose synthesis as long as blood glucose was maintained at normal levels. The results therefore indicate that the glucose turnover in mammary glands, which in high yielding dairy ruminants represents the main organ of glucose utilization in the body, is in fact independent of changes in plasma insulin concentrations.

The glucose uptake of the mammary gland accounts for 60-85 per cent of the glucose utilization in lactating dairy ruminants. Two thirds or more of the mammary uptake is converted to lactose (Annison and Linzell 1964; Bickerstaffe, Annison and Linzell 1974). The role played by insulin in this extensive carbohydrate uptake and conversion is, however, not fully understood. Insulin is essential for the maintenance of secretory activity in mammary tissue in rodents (Walters and MacLean 1968; Martin and Baldwin 1971a, b). Lack of insulin for 36-48 h or more reduces milk secretion and enzymatic activity in the mammary gland. In goats, alloxan diabetes of some weeks duration causes complete and irreversible loss of secretory activity (Nowak and Dzialoszynski 1967). In contrast, the acute effects of insulin deficiency on milk secretion are negligible (Hove 1978).

The effects of increased insulin levels on mammary gland metabolism and secretion rate have been studied both *in vivo* and *in vitro* Bauman *et al.* (1973) and Baldwin *et al.* (quoted

h (Baldin and Louis 1975) have shown that insulin, when added to slices of cow mammary gland, has no effect on lipogenesis from acetate or on the pattern of glucose metabolism. As routine administration of insulin to lactating ruminants reduces both milk volume and lactose production (Gowen and Tobey 1932), but these changes seem to be secondary to the hypoglycemia developed by insulin since they may be prevented by simultaneous intravenous infusions of glucose.

Since marked fluctuations in plasma insulin occur throughout the day (McAtee and Tinkler 1971, Hove and Blom 1973, Hart *et al.* 1975) while milk production is almost constant from hour to hour, one might expect that mammary glucose uptake and carbohydrate secretion proceeds independently of the changing concentrations of insulin in blood. To test this two series of insulin infusion experiments were designed using local infusions into a mammary artery. In the first series the effects of increased plasma insulin concentrations on lactose secretion were studied during close arterial insulin infusion. In the second series the hypoglycemic conditions resulting from insulin infusion in series one were offset by simultaneous infusions of glucose to maintain normoglycemic conditions. Mammary blood flow, glucose extraction and lactose secretion were measured.

Methods

Animals

Adult goats weighing 30–40 kg are used for the study. The animals were in mid to late lactation and yielded from 1 to 1.5 kg milk daily. The goats were housed indoors in individual pens during the experiments, and were fed hay and pelleted concentrate ration containing 12.5 per cent crude protein *ad lib.* Feed was given twice daily at about 8 a.m. and 4 p.m. and milking was performed in connection with the meals. Feeding and milking were carried out as usual on days of experiment. The goats are allowed to be milked for 1–2 h before the experiments started.

Surgical preparation

1. Two goats are prepared for local intra-arterial (i.a.) infusions while having the mammary glands intact. All vessels crossing the midline between the two halves of the udder are resected. In addition mammary artery is exteriorized (Lunnell 1960). To provide access to the arterial circulation the gland, the external pudic artery is exteriorized in a skin-covered loop. The artery is carefully freed from its accompanying nerves and vessels, side branches were ligated and the vessel exteriorized either close to the midline or on the crano-lateral surface of the udder. The pudic artery loop did not work satisfactorily for the time needed for experimentation, partly due to an increasing tendency to spasm contractions, and partly due to the development of collateral arterial supply. To overcome these difficulties mammary anastomoses are performed.

2. In the additional goats one of the mammary glands is auto-transplanted to the neck according to the method of Lunnell (1963). The external pudic artery was anastomosed end to end with the left carotid artery and the external pudic vein side to side with the jugular vein. The carotid artery is exteriorized in a skin-covered loop about 5 cm proximal to the anastomosis.

Experimental design

The series of infusions were performed, one of 5 and the other of 10 h duration. The goats were milked after the intravenous injection of 100 mU of oxytocin. The first 2 h thereafter were used as control period. This follows a 3 or 8 h test period. The first 2 h were used to measure the basal or pre-infusion milk production. During this period infusions of saline were usually given. Thereafter the effects of insulin and/or glucose infusions were tested for 3 or 8 h.

The validity of the present method for studying milk production depends on the assumption that changes in mammary metabolism induced by the infusions can be detected during the infusion period. The increased lactose production may be influenced by lactose synthetase but not milked out during the foregoi g

hours. In the perfused mammary gland preparation Hardwick & Linzell (1960) have calculated that the amounts of lactose remaining in a gland after oxytocin-aided emptying is less than one hour's normal lactose production. Changes in lactose secretion occurring during the last 2 h of a 3 h infusion therefore most probably reflect a true change in mammary production of lactose and not remaining lactose produced earlier. For the eight hour infusions, this should obviously be the case.

In accordance with the results of Linzell (1967*a, b*) and Linzell and Peaker (1971) hourly lactose secretion was found to be nearly constant during several 5 h periods of measurements undertaken to establish spontaneous variations in lactose secretion rate. A total of 11 periods were studied in 6 goats which hourly yields ranging from 40–120 ml. When the average secretion during the two first hours was set at 100 per cent lactose secretion during the next 3 h was 100 ± 9 , 103 ± 8 and $103 \pm 11\%$ (mean and S.D.), respectively. As a consequence hourly milking was used in the 3 h infusion experiments. When oxytocin-aided milking was continued for periods of up to 10 h, Linzell and Peaker (1971) observed increases in milk yield and slight changes in milk composition. To minimize these effects of the oxytocin injections, the mammary glands were left undisturbed for the first 6 h of the 8 h insulin infusion.

During a preliminary series of expts. it was found that elevations of plasma insulin concentrations to 2.5–4 $\mu\text{g/ml}$ in the systemic circulation invariably led to hypoglycaemia and reduced milk production within 2–3 h. Thus, to ensure a proper stimulation of the possibly insulin sensitive processes in the mammary gland in the present study the dose of insulin infused locally to the mammary arteries were adjusted so as to give blood concentration in the gland of at least 3 μg insulin per ml.

Series 1. Short term (3 h) insulin infusions. Insulin (porcine Leo[®]-neutral) diluted in saline was infused at a rate of 2 $\mu\text{g/kg h}$ to the exteriorized mammary artery on 5 occasions in 4 goats. The lactate was given through a 1.0 mm (o.d.) plastic catheter (Venflon[®]) previously inserted into the artery. Insulin concentrations in the desired range were obtained in the mammary vein, but simultaneously the arterial plasma glucose concentrations decreased considerably.

Series 2. Long term (8 h) infusions of insulin and glucose. Insulin was infused locally to the mammary artery (2 $\mu\text{g/kg h}$) for 8 h. Simultaneously glucose was infused to the contralateral jugular vein at a rate of approximately 2 mg/kg min to maintain normoglycaemia. The infusion rate was adjusted according to variations in the concentration of glucose during the expt. Two expts. with each of the two goats with autotransplanted glands were carried out.

Milking. Intravenous injection of 100 mU of synthetic oxytocin was given to evoke milk ejection. Five minutes elapsed from oxytocin administration to the completion of milking. The goats were milked by hand and the volumes from the two udder halves were measured separately. Aliquots of 5 ml were taken after thorough mixing, cooled on ice during the expt. and then stored at -20°C until analysed.

Blood sampling. Catheters for blood sampling (o.d. 1.0 mm) were inserted into the carotid or mammary artery and mammary venous loops under local anaesthesia. In the two goats with mammary glands in situ the external podic veins were compressed manually during venous sampling to force venous blood to leave the gland through the caudal epigastric (milk) vein where the sampling catheters were located (Linzell 1960). Mammary venous blood from the autotransplanted gland was sampled from the jugular vein after compression of the vein distal to the anastomosis with the mammary vein. About 2 ml of blood was withdrawn each time. After prompt centrifugation in heparinized tubes the plasma was removed and kept on ice until termination of the expt. Plasma was then frozen and kept at -20°C until analysed. The catheters were filled with heparinized saline (5 IU/ml) between samplings. Two pairs of arterial and venous blood samples were taken every hour, 20 and 30 min after each oxytocin injection.

Mammary blood flow was measured in the goats with autotransplanted glands by means of an electromagnetic flow meter (Nycotron 376). A cuff type probe (5 or 6 mm I.d.) was chronically implanted around the carotid artery 5–8 cm proximal to the loop some weeks prior to the beginning of the expts. The leads were tunneled under the skin and taken out on the middle of the neck. The probes remained in position and worked well for several months. Zero blood flow was obtained by clamping of the carotid loop distal to the probe. The zero readings were repeatedly checked during the experiments. The flow signal from the probe was calibrated at the start and at the end of each experiment using a digital integrator (Nycotron 394). 20 ml of blood was withdrawn by syringe from the clamped artery and the integrated flow signal used to relate instrument output to actual flow values. Usually this calibration procedure was repeated 3 to 5 times at the beginning and end of the expts. The coefficient of variation of the calibration procedure ranged between 4 and 10% with typical values of 6–7%. The flow signals were recorded on a moving chart and average hourly flow rates calculated afterwards.

Plasma glucose, insulin and milk lactose were measured as reported earlier (Hove 1978 in press).

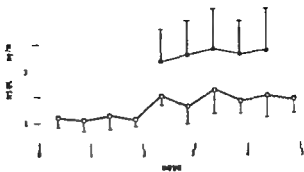


Fig. 1 a.

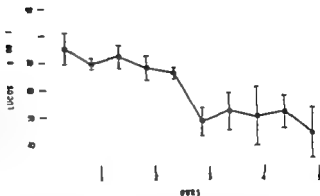


Fig. 1 b.

Fig. 1. (a) Arterial (O) and mammary veins (●) insulin concentrations before and during the intrarterial infusion of insulin doses 1. (b) Arterial glucose concentrations. Infusion indicated by black bar. Means and S.D. (5 pairs).

Results: Student's *t*-test for paired comparisons was used to evaluate changes in lactose secretion. The glucose values in the preinfusion periods are compared with the secretion during the last 14 hours of the infusion both in the short and long-term infusion experiments. $P < 0.05$ was taken to represent statistical significance.

Results

Series 1: Short term insulin infusions. The infusion of insulin into the artery of one of the mammary glands—either *in situ* or autotransplanted—raised the insulin concentration in mammary venous plasma to between 3.5 and 4 ng/ml (Fig. 1 a). The plasma levels obtained in the mammary veins were significantly higher than the insulin concentrations in the systemic circulation ($p < 0.05$).

The systemic arterial insulin concentration, and hence the insulin concentration in the blood perfusing the other gland, rose from 1.25 before the expt. to about 2 ng/ml at the end of the infusion (Fig. 1 a). The infusion of insulin resulted in a decrease of the arterial plasma glucose by 15–20 mg/100 ml to about 50 mg/100 ml (Fig. 1 b). A reduction in lactose secretion

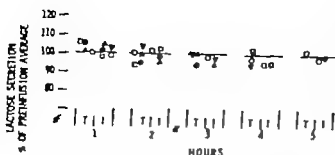


Fig. 2. Lactose secretion before and during the 1 a. infusion of insulin *in situ*. 1 Lactose secretion in per cent of the average secretion for each gland during the two-hour pre-infusion period. T and U Transplanted gland and the gland remaining *in situ* respectively. In the goat with both glands *in situ* T represents the infused gland. Black bar indicates the period of infusion to T

tion was observed during the last two hours in most glands (Fig. 7). No differences between infused and non-infused glands could be detected. Thus when all observations of individual gland results were combined, an average reduction of lactose yield to 91 per cent of the pre infusion yield was observed ($p < 0.02$)

Series 2 Long term infusions of insulin and glucose In these experiments plasma insulin concentrations in mammary venous blood reached a plateau level of about 4.5 ng/ml while systemic insulin concentrations increased from about 1 to between 2.5 and 3 ng/ml (Fig. 3a). The considerable increase in systemic insulin was counter balanced fairly well by the *in situ* venous infusion of glucose, thus avoiding the development of the hypoglycemia observed during the short term infusion (Fig. 3b)

Lactose secretion remained at a constant level for the complete infusion period. No changes in lactose secretion could be observed between the infused, transplanted glands and the glands remaining *in situ* (Fig. 4)

Mammary glucose uptake was calculated from plasma flow and arteriovenous plasma glucose differences. Since lactose secretion rates and glucose uptakes were practically identical in the two goats during this series of the experiment, actual uptake figures are presented in Fig. 5. Although the figures for glucose uptake differed significantly for some of the one hour periods, averages for pre infusion and infusion periods were practically identical (30.9 as compared to 31.2 mg/min). The lactose secretion from the glands accounted for 80.5 and 82.0 per cent of the average glucose uptake in the pre-infusion and insulin infusion periods respectively. Mammary blood flow increased throughout the experiments in both goats. 30–60 per cent higher values were observed at the conclusion of the infusions than in the pre-infusion period. Concomitantly a similar reduction in α -D differences of glucose occurred, thus giving relatively constant glucose uptake figures. Milk yields of about 0.6 ml/min were recorded during this series of experiments, and the average ratios of blood flow to milk secretion were between 520 and 670.

Discussion

The infusion of insulin into the mammary artery gave rise to average mammary vein insulin concentrations between 3.5 and 4.5 ng/ml and systemic insulin concentrations of about 2 ng/ml. The infusion further resulted in a reduction of about 20 mg/100 ml in plasma glucose. Glucose seems to be a rate limiting substrate for milk synthesis when available in sub-

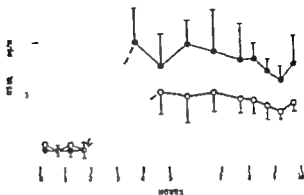


Fig. 3 a.

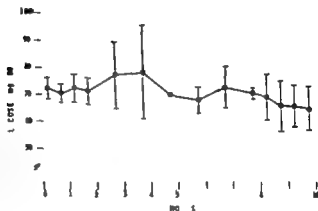


Fig. 3 b.

Fig. 3 (a) Arterial (O) and coronary (●) lactate concentrations before and during the 12 h infusion of insulin (□) and glucose (■) (b) Arterial glucose concentration. Infusion indicated by black bar. Means and S.D. (2 goats in each of 2 goats).

optimal amounts (Hardwick *et al.* 1961; Kronfeld *et al.* 1963; Rook and Hopwood 1970). The reduction in mammary lactose secretion observed during the last part of the insulin infusion most probably therefore resulted from the reduced plasma glucose levels. In fact, the effects of the hypoglycaemia which developed during the first hour of the infusion were clearly evident on the lactose secretion after the second hour. This rapid decrease in lactose secretion emphasizes the need for a continuous supply of glucose to maintain a constant rate of mammary lactose synthesis.

When in the combined infusion experiments the systemic effects of insulin on plasma glucose were offset by i. glucose, no change in mammary glucose uptake or lactose secretion could be observed. These findings should be compared to the results of a series of expts. where alloxan diabetic lactating goats were controlled by insulin infusions so as to maintain normal blood glucose concentrations (Hove 1978). When insulin treatment was discontinued plasma insulin declined to very low levels and prominent signs of insulin

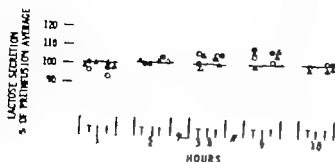


Fig. 4 Lactose secretion before and during the 8 h infusion of insulin (I.) and glucose (L.v.). Text as in Fig. 2.

deficiency developed in the systemic circulation in the course of 1-2 h. Mammary lactose secretion continued in an unchanged manner for 4 h after withdrawal of the insulin treatment, however. It therefore appears that the mammary secretion of lactose seems to be unaffected by short term (4-8 h) changes in the concentration of plasma insulin. Alternatively the possibility exists that the hyperinsulinism per se could induce endocrine changes which would interfere with or even mask the effects of insulin. Little is known, however about the hour to hour regulation of mammary metabolism during established lactation. Increased plasma levels of catecholamines might interfere with the mammary substrate supply by inducing vasoconstriction. In the present expts. constant or increasing mammary blood flows during the infusion period seem to exclude this possibility. Although changes in plasma levels of prolactin probably occur as a result of the experimental procedures (Mikkelsen, Bryant *et al.* 1970) most evidence seems to indicate that changes in plasma prolactin is without effect on milk yields during an established lactation (Cowie 1971, Karg *et al.* 1972, Hart 1973). Hart and Flux (1973) have demonstrated that increases in the plasma concentration of growth hormone may occur in the goat after milking. The possible role of this response in regulating mammary metabolism remains unclear, however.

The findings of blood to milk flow ratios between 500 and 700 is in accordance with figures obtained by thermodilution flow measurements in cow and goat (Linzell 1960, Bickerstaffe *et al.* 1974). The amount of glucose accounted for by lactose secretion (80-82 per cent) is within the upper range of what has been reported earlier (Andersen and Linzell 1964). Glucose oxidation or output as milk fat glycerol were not measured in the present study but it seems probable that these other processes utilizing glucose were proceeding.

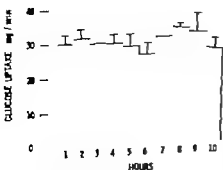


Fig. 5 Mammary glucose uptake in the transplanted gland before and during the 8 h infusion of insulin (I.) and glucose (L.v.). Infusion indicated by black bar. Means and S.D. of 2 expts. in each of 2 goats.

about the same rate in the pre-infusion and infusion periods, when the lack of effects of hyperinsulinaemia on glucose uptake and lactose secretion is recalled.

Since the changes in plasma insulin concentration observed in connection with feeding such is of rather short duration ($t = 4-6$ h) and of smaller magnitude than those imposed by the present infusions, the conclusion can be drawn that the changes in plasma insulin which occurs during ordinary feeding conditions probably do not participate directly in the regulation of mammary carbohydrate uptake and secretion. Numerous studies have stressed the importance of the mammary gland in the utilization of available glucose. In the goat Amos and Litzell (1964) have demonstrated that 60-85 per cent of the glucose entry into a udder by the mammary gland, and comparable figures have been obtained by others in the lactating cow (Bickerstaffe *et al.* 1974; Patterson and Litzell 1974). The implications of the present findings would be that the major outflow of glucose during lactation in ruminants is independent of short term fluctuations in plasma insulin concentrations.

The skilled technical assistance of Mr Per Moen is acknowledged. The study was supported by the Norwegian Agricultural Research Council.

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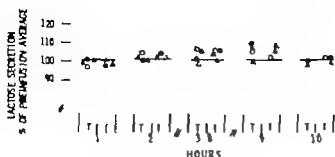


Fig. 4 Lactose secretion before and during the 8 h infusion of insulin (Ia) and glucose (I.). Text as in Fig. 2.

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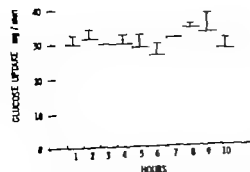


Fig. 5 Mammary glucose uptake by the transplacental gland before and during the 8 h infusion of insulin (Ia) and glucose (I.). Infusion indicated by black bar. Mean and S.D. of 2 experiments, each of 2 goats.

that the same rate in the pre-infusion and infusion periods, when the lack of effects of sympathomimetics on glucose uptake and lactose secretion is recalled.

Since the changes in plasma insulin concentration observed in connection with feeding only a relatively short duration (i.e. 4-6 h) and of smaller magnitude than those imposed by the present infusions, the conclusion can be drawn that the changes in plasma insulin which occur during ordinary feeding conditions probably do not participate directly in the pattern of mammary carbohydrate uptake and secretion. Numerous studies have stressed the importance of the mammary gland in the utilization of available glucose. In the goat Mann and Linzell (1964) have demonstrated that 60-85 per cent of the glucose entry is used by the mammary gland, and comparable figures have been obtained by others in the lactating cow (Buckner et al. 1974; Patterson and Linzell 1974). The implications of the present findings would be that the major outflow of glucose during lactation in mammals is independent of short term fluctuations in plasma insulin concentrations.

Technical assistance of M. Per Moen is acknowledged. The study was supported by the Norwegian Agricultural Research Council.

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The distribution and secretion of kallikrein in some exocrine organs of the rat

By

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Abstract

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soluble kallikrein as quantitated in the submandibular, sublingual, and parotid glands and in the skin. No kallikrein was detected in the orbital lacrimal glands and uvea. The highest kallikrein concentrations (EU/ml) were in all major salivary gland secretions seen after α -adrenergic stimulation, less after denervate and least after perivascular sympathetic stimulation. When taking into account the large variations in activity, α -adrenergic stimulation was in the parotid and particularly in the submandibular gland found to result in the highest kallikrein secretory activity measured by the kallikrein secretory rate (EU/h). This shows that the kallikrein-rich granular tubular cells are selectively activated through α -adrenergic receptors. The differences observed in the parotid saliva were small and not always statistically significant. However, less cervical nerve stimulation was superimposed upon parasympathetic stimulation, thus secretory rate as well as kallikrein concentration increased. The large individual variations in salivary gland kallikrein content and secretion and the rather small differences observed in kallikrein activity rate after nervous stimulation of the parotid and sublingual glands, may indicate that the kallikrein-containing striated ducts are also influenced by factors other than the secretory nerves. The kallikrein activities and secretory rate in urine was studied. A strong positive correlation between kallikrein activity rate and fluid volume was found in urine but not in saliva.

Glandular kallikreins are serine proteinases (E.C.3.4.21.8) able to release kinins from kininogen. In mammals they are found in the kidney and in exocrine glands as well as in the secretions of these organs. In the major salivary glands of the rat kallikrein is confined to the duct system (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976, Ørstavik *et al.* 1977, Ørstavik 1978). Regulation of glandular kallikrein secretion has previously been investigated in the rat submandibular gland (Ørstavik and Gautvik 1977). However, this gland differs from the other major salivary glands by the presence of granular tubules, a structure particularly rich in kallikrein (Ørstavik *et al.* 1977). Therefore, interpretations on kallikrein function in general must be based on the stimulatory pattern of this gland alone. This report describes an extended study of the kallikrein secretion in the major salivary glands of the rat. Furthermore, a comparison of enzyme content, cellular localization, and kallikrein secretion is made among various kallikrein-containing organs.

Materials and Methods

Animals Female Sprague Dawley rats were used (14–16 weeks, 250–300 g b.wt.). They were given tap water and food ad lib. The animals were anesthetized with intraperitoneal injections of pentobarbital (70 mg/kg b.wt.). Animals used for collection of saliva were tracheotomized and maintained spontaneous respiration during the experimental period. Those used for collection of urine were kept in metabolic cages and were fed on ground pellets and water ad lib.

Gland homogenates The parotid, submandibular and sublingual salivary glands, and the exorbital lacrimal glands were dissected out and frozen at -20°C until used within about 4 weeks. After thawing, fat and capsular material were removed, the glands were cut into pieces, suspended in PBS (0.01 M Na-phosphate buffer pH 7.4 and 0.15 M NaCl), 1:10 wt./vol., and homogenized in a Potter Elvehjem homogenizer (40 strokes, 4°C). After centrifugation (20 000 g 20 min, 4°C) the supernatant fluid was collected and frozen (-20°C) for subsequent measurements. The sediment of the submandibular gland homogenate was re-homogenized twice in the same volume of buffer and the 3 supernatant fluids were combined. Due to the small size of the sublingual gland 4 or 6 glands were pooled to give one homogenate, and 4 different homogenates were made. With the other organs one homogenate was produced for each gland.

Collection of saliva The main excretory ducts of the parotid, sublingual and submandibular glands are cannulated with a polyvinyl ethylene tubing (PP 10 Portex), which was pulled out when used for the two first. The parotid duct was dissected free from the masseteric muscle and cannulated distal to the point where it leaves the marginal mandibular branch of the facial nerve. The ducts of the submandibular and sublingual glands were exposed by removal of the mylohyoid and digastric muscles. The two ducts were carefully separated under a dissecting microscope. The submandibular duct was medially situated to the sublingual duct and showed a larger lumen. The latter was also recognized by its content of mucous and thread-drawing saliva. Retrograde injection of tryptophane blue solution was occasionally performed after the fluid collection to control the cannulation. In each animal the parotid glands were cannulated bilaterally whereas for the submandibular and sublingual glands only one of the two was cannulated on the same side. Saliva was collected during the gland activation period into tartrated tubes, and the volume of saliva was estimated by weight (1 μl = 1 mg).

Stimulation of salivary gland secretion Parasympathetic stimulation was obtained by (1) injections of pilocarpine (0.4 ml, 4 g/l) into the femoral vein, (2) injection of acetylcholine (0.1 ml, 0.1 g/l) into the femoral vein, (3) electrical stimulation of the auriculotemporal nerve activating the parotid gland or of the nerve plexus surrounding the ducts of the submandibular and sublingual glands (7.5 V 2 ms duration and 9.5 Hz). Sympathetic stimulation was performed by (a) intermittent electrical stimulation of the sympathetic cervical nerve (7.5 V 2 ms duration and 9.5 Hz), (b) injection of norepinephrine (0.06 ml, 1 g/l) into the femoral vein or (c) injection of isoproterenol (0.15 ml, 1 g/l) also into the femoral vein. Saliva was usually collected for 10–15 min or after norepinephrine administration until the rat died from pulmonary edema.

Collection of urine The rats were kept in metabolic cages for 2 days before the collection started. Urine was then accumulated during periods of 24 h for 15 days. Toluene was added to the collecting glass to prevent bacterial growth. The urine was centrifuged (10 000 g 20 min, 4°C), and frozen (-20°C).

Collection of tears Tears were collected from the corneal surface of one rat after electrical stimulation of the temporal nerve for about 2 min (7.5 V 2 ms duration, 9.5 Hz). Attempts to cannulate the duct of the exorbital lacrimal gland were unsuccessful.

Kallikrein measurement Kallikrein was quantified as described previously (Ørstavik *et al.* 1977) by its antigenicity in single radial immunodiffusion system and by its Bz-Arg-OEt-esterase activity. For the exorbital lacrimal gland homogenates and the tears 10–15 μl were incubated for up to 4 h at 37°C with the Bz-Arg-OEt-substrate. A small volume of buffer was added to the sublingual gland saliva before it was frozen to prevent desiccation, and therefore this secretion also had to be incubated for 4 h. All other samples were incubated from 5 to 60 min in a dilution providing a measurable breakdown rate of the substrate.

The rabbit serum to submandibular gland kallikrein used in the BRID-measurements contained 32 precipitating units when tested by double diffusion against standard submandibular gland homogenate (0.19 g kallikrein and 2.1 g protein/l).

Statistical analyses. The results are given as mean values \pm S.D. Differences between experimental groups were examined by the non-paired, two-tailed Wilcoxon test for non-parametric data (van Elteren 1960).

Drugs. Pentobarbital (Nembutal sodium® Abbott Laboratories, London), pilocarpine (NAF Laboratories A/S, Oslo, Norway), acetylcholine chloride (F Hoffmann-La Roche & Co. A.G. Basel), norepinephrine (Nor-adrenalin® Norsk Astra A/S, Oslo, Norway), isoproterenol (Isoprenalini sulphat® Norwegian Medical Depot, Oslo, Norway).

TABLE I. Quantification of kallikrein antigen and Bz-Arg-OEt-esterase activity in homogenates of rat exocrine glands.

	Number of glands	Kallikrein antigen ($\mu\text{g/g}$) ^a	Bz-Arg-OEt-esterase activity		esterase activity representing kallikrein ^b
			(EU/gland)	(EU/g) ^c	
Submandibular gland	34	11,950 \pm 2,253	2,101 \pm 682 ^d	9,578 \pm 384	78 \pm 16 ^e
Sublingual gland	4	126 \pm 5	3.9 \pm 0.5	117 \pm 28	70 \pm 9
Parotid gland	3	Below detection limit	0.8 \pm 0.5	3.9 \pm 2.5	
Exorbital lacr. gl.	6	Absent or below detection limit			
Pancreas (not act.) ^f	8	132 \pm 51	0	0	

^a Concentration on the basis of glandular wt. or weight.

^b The percentage of total Bz-Arg-OEt-esterase activity representing kallikrein is calculated individually for each gland using the specific esterase activity of purified submandibular gland kallikrein, (599 EU/mg (Broadbent et al. 1976)).

^c Data obtained from Orstavik and Gjessvik (1977).

^d The values were obtained from Orstavik and Gjessvik (1978). In the pancreas glandular kallikrein is found as pro-enzyme. Therefore, no kallikrein esterase activity is detected in non-activated gland homogenates.

Results

Quantification of kallikrein in gland homogenates. The concentration of kallikrein in the various exocrine organs of the rat is given in Table I. In the parotid gland the kallikrein concentration was too low to be detected by the SRID. No esterase activity was found in the non-activated pancreas homogenates where kallikrein is found as a pro-enzyme (Orstavik and Gjessvik 1978). In the exorbital lacrimal gland neither kallikrein antigen nor Bz-Arg-OEt-esterase activity was detected.

Salivary flow rate. Resting saliva was only seen in the sublingual gland and then only in very minute quantities. Parasympathetic stimulation induced a rich flow of saliva from all of the major salivary glands (Table II). The flow rate from the submandibular gland decreased during the stimulation period, as did the pilocarpine induced secretion from the parotid gland. Salivary secretion following stimulation of the auricular temporal nerve most often showed a temporary increase during the stimulation period. Sympathetic stimulation also produced saliva from all three glands although with lower secretory rate than after parasympathetically stimulation (Table II), and with a decrease in flow rate during the stimulation period. In the sublingual gland salivary flow rate induced by sympathetic stimulation was very low (Table II).

Stimulation of salivary kallikrein secretion. The concentration of kallikrein in saliva and its secretory rate from the submandibular, sublingual, and parotid glands are given in Table II. The kallikrein concentration of the parasympathetically induced saliva was for all three glands significantly ($P < 0.01$) lower than that produced by the various adrenergic stimulation procedures.

In the submandibular gland kallikrein secretion was strikingly released by α -adrenergic stimulation (norepinephrine). Also β -adrenergic stimulation (isoproterenol) resulted in some kallikrein secretion giving a higher salivary kallikrein concentration although much lower

Materials and Methods

Animals. Female Sprague-Dawley rats were used (14–16 weeks, 250–300 g b.w.t.). They were given tap water and food ad lib. The animals were anesthetized with intraperitoneal injections of pentobarbital (70 mg/kg b.w.t.). Animals used for collection of saliva were tracheotomized and maintained spontaneous respiration during the experimental period. Those used for collection of urine were kept in metabolic cages and excreted on ground pellets and water ad lib.

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Stimulation of salivary gland secretion. Parasympathetic stimulation was obtained by (a) injections of pilocarpine (0.4 ml, 4 g/l) into the femoral vein, (b) injection of acetylcholine (0.1 ml, 0.1 g/l) into the femoral vein, (c) electrical stimulation of the a. neulotemporal nerve activating the parotid gland or of the nerve plexus surrounding the ducts of the submandibular and sublingual glands (7.5 V, 2 ms duration and 9.5 Hz). Sympathetic stimulation was performed by (a) intermittent electrical stimulation of the sympathetic cervical nerve (7.5 V, 2 ms duration and 9.5 Hz), (b) injection of norepinephrine (0.06 ml, 1 g/l) into the femoral vein, or (c) injection of isoproterenol (0.15 ml, 1 g/l) also into the femoral vein. Saliva was usually collected for 10–15 min or after norepinephrine administration until the rat died from pulmonary edema.

Collection of urine. The rats were kept in metabolic cages for 2 days before the collection started. Urine was then accumulated during periods of 24 h for 15 days. Toluene was added to the collecting glass to prevent bacterial growth. The urine was centrifuged (10 000 g, 20 min, 4°C), and frozen (-20°C).

Collection of tears. Tears were collected from the corneal surface of one rat after electrical stimulation of the temporal nerve for about 2 min (7.5 V, 2 ms duration, 9.5 Hz). Attempts to cannulate the duct of the exorbital lacrimal gland were unsuccessful.

Kallikrein measurements. Kallikrein was quantified as described previously (Orstavik *et al.* 1977) by its antigenicity in a single radial immunodiffusion system and by its Bz-Arg-OEt-esterase activity. For the exorbital lacrimal gland homogenates and the tears 10–15 μl were incubated for up to 4 h at 37°C with the Bz-Arg-OEt-substrate. A small volume of buffer was added to the sublingual gland saliva before it was frozen to prevent desiccation, and therefore this secretion also had to be incubated for 4 h. All other samples were incubated from 5 to 60 min in a dilution providing a measurable breakdown rate of the substrate.

The rabbit antiserum to submandibular gland kallikrein used in the SRID-measurements contained 32 precipitating units when tested by double diffusion against a standard submandibular gland homogenate (0.19 g kallikrein and 2.1 g protein/l).

Statistical analyses. The results are given as mean values \pm S.D. Differences between experimental groups were examined by the non-paired two-tailed Wilcoxon test or non-parametric data (vs. Elterén 1960).

Drugs. Pentobarbital (Nembutal sodium® Abbott Laboratories, London), pilocarpine (NAF-Laboratories A/S, Oslo, Norway), acetylcholine chloride (F. Hoffmann-La Roche & Co. A.G. Basel), noradrenaline (Nor-adrenalin® Norsk Astra A/S, Oslo, Norway), isoproterenol (Isoprenalin® sulphate® Norwegan Medical Depot, Oslo, Norway).

TABLE I Quantification of kallikrein antigen and H₂-Arg-OEt-esterase activity in homogenates of rat exocrine glands.

	Number of glands	Kallikrein antigen ($\mu\text{g}/\text{g}^{\text{w}}$)	H ₂ -Arg-OEt-esterase activity		esterase activity representing kallikrein
			(EU/gland)	(EU/g ^w)	
Submandibular gland	14	$11\,950 \pm 2\,253$	$2\,101 \pm 482^{\text{a}}$	$9\,578 \pm 2\,384$	$78 \pm 16^{\text{a}}$
Sublingual gland	4	126 ± 5	$3\,9 \pm 0.5$	117 ± 23	70 ± 9
Parotid gland	3	Below detection limit	0.3 ± 0.5	3.9 ± 2.5	
Exorbital lacr. gl.	6	Absent or below detection limit			
Pancreas (not act.) ^d	8	132 ± 51	0	0	

^a Concentration on the basis of glandular wt. (right).

The percentage of total H₂-Arg-OEt-esterase activity representing kallikrein is calculated individually for each gland using the specific esterase activity of purified submandibular gland kallikrein, 1/399 EU/ μg (Brackley *et al.* 1976).

^b Data obtained from Ørstavik and Glemser (1977).

^c The values were obtained from Ørstavik and Glemser (1978). In the pancreas glandular kallikrein is found as a pro-enzyme. Therefore, no kallikrein esterase activity is detected in non-activated gland homogenates.

Results

Quantification of kallikrein in gland homogenates. The concentration of kallikrein in the various exocrine organs of the rat is given in Table I. In the parotid gland the kallikrein concentration is too low to be detected by the SRID. No esterase activity was found in the non-activated pancreas homogenates where kallikrein is found as a pro-enzyme (Ørstavik and Glemser 1978). In the exorbital lacrimal gland neither kallikrein antigen nor H₂-Arg-OEt-esterase activity was detected.

Salivary flow rate. Resting saliva was only seen in the sublingual gland and then only in very minute quantities. Parasympathetic stimulation induced a rich flow of saliva from all of the major salivary glands (Table II). The flow rate from the submandibular gland decreased during the stimulation period, as did the pilocarpine induced secretion from the parotid gland. Salivary secretion following stimulation of the auricular temporal nerve most often showed a temporary increase during the stimulation period. Sympathetic stimulation also produced saliva from all three glands although with a lower secretory rate than after parasympathetically stimulation (Table II), and with a decrease in flow rate during the stimulation period. In the sublingual gland salivary flow rate induced by sympathetic stimulation was very low (Table II).

Stimulation of salivary kallikrein secretion. The concentration of kallikrein in saliva and its secretory rate from the submandibular, sublingual, and parotid glands are given in Table III. The kallikrein concentration of the parasympathetically induced saliva was for all three glands significantly ($P < 0.01$) lower than that produced by the various adrenergic stimulation procedures.

In the submandibular gland kallikrein secretion was strikingly released by α -adrenergic stimulation (norepinephrine). Also, β -adrenergic stimulation (isoproterenol) resulted in some kallikrein secretion giving a higher salivary kallikrein concentration although much lower

TABLE II Salivary flow rate from the submandibular, sublingual, and parotid glands following parasympathetic and sympathetic stimulation.

Stimulation	Submandibular gland (0.216 ± 0.023 g) ^a				Sublingual gland (0.037 ± 0.004 g) ^a				Parotid gland (0.214 ± 0.046 g) ^a			
	µl/min	µl/min/ g.w. ^b	n	min	µl/min	µl/min/ g.w. ^b	n	min ^c	µl/min	µl/min/ g.w. ^b	n	min ^c
<i>Parasympathetic</i>												
Electrical	17.0 ± 9.6	78.7	6	10-30	1.1 ± 0.7	29.7	3	10-14	8.3 ± 3.2	38.8	5	10
Pilocarpine	7.1 ± 2.6	32.9	5	20-30	3.6 ± 0.7	97.3	7	15	7.8 ± 3.0	36.4	15	6-30
Acetylcholine	8.7	40.3	2	20	0.3	8.1	1	2	2.8 ± 2.8	13.1	4	2-30
<i>Sympathetic</i>												
Cervical nerve	4.4 ± 1.4	20.3	13	3-15	0.07 (0.03-0.12)	1.9	3	6-10	1.8 ± 1.3	8.4	15	2-15
Norepinephrine	1.9 ± 0.9	8.8	5	3-15	0.03 (0.01-0.04)	0.8	3	5-15	1.3 ± 1.1	6.1	7	5-15
α-adrenergic	2.6 ± 2.0	12.0	7	5-20								
Isoproterenol	0.8 ± 0.6	3.7	6	20-40	0.05 ± 0.04	1.4	4	15-16	1.4 ± 0.6	6.5	8	15-36
β-adrenergic	1.5 ± 0.5	6.9	5	15-30								

^a Wet weight of the glands.^b Salivary flow rate related to the wet weight of the glands.^c The length of the stimulation period.

α-adrenergic = cervical nerve stimulation with the addition of a β-blocker (propranolol).

β-adrenergic = cervical nerve stimulation with the addition of an α-blocker (phenolamine).

n = number of glands when only three glands were used the range of results is given in parentheses below the mean.

than after α-adrenergic stimulation. Parasympathetic saliva contained comparably little kallikrein although of the same concentration order as parotid saliva. The high α-adrenergic release of kallikrein was a true adrenergic induced kallikrein secretory response not due to the lower salivary flow rate following norepinephrine stimulation since also the kallikrein secretory rate, EU/min, was very high (Table III). The kallikrein secretory rate following β-adrenergic (isoproterenol) stimulation, on the other hand, did not differ much from that seen after parasympathetic stimulation. Electrical stimulation along the submandibular gland duct resulted in a saliva with a somewhat higher kallikrein concentration ($P < 0.10$) and a higher kallikrein secretory rate ($P < 0.01$) than pilocarpine stimulation.

In the parotid gland, like in the submandibular gland, sympathetic stimulation resulted in a higher kallikrein concentration than did parasympathetic stimulation ($P < 0.01$). The acetylcholine induced parotid saliva was an exception with a remarkably high kallikrein concentration (Table III). The difference between parasympathetically and sympathetically stimulated saliva was not significantly valid when taking into account the differences in salivary flow rate, although the same tendency was observed (Table III). However, when in the parotid gland sympathetic stimulation was superimposed on parasympathetic stimulation, kallikrein secretory rate as well as kallikrein concentration increased. In the two animals investigated (Fig. 1a and b), α-adrenergically stimulated saliva had a significantly higher kallikrein concentration ($P < 0.05$) and secretory rate ($P < 0.01$) than did β-adrenergic parotid

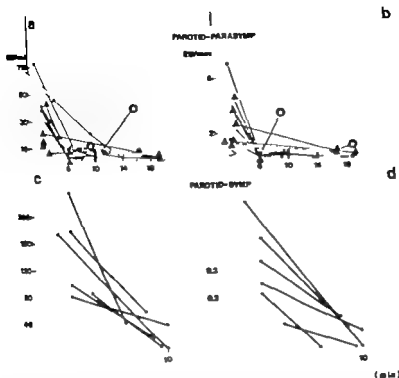


Fig 1 Salivary kallikrein concentration (a and c) and secretory rate (b and d) during the period of parasympathetic (auriculotemporal nerve \circ and pilocarpine Δ) and sympathetic (cervical nerve) stimulation of the parotid gland. Stimulation of the sympathetic cervical chain was superimposed on parasympathetic stimulation \square and resulted in increased kallikrein concentration (a) as well as kallikrein secretory rate (b).

saliva. For the sublingual gland saliva similar pattern in kallikrein concentration as that for the parotid gland was seen. However in this gland no differences could be observed in the kallikrein secretory rates.

Individual variations in kallikrein secretion. The kallikrein concentration and secretory rate varied greatly from animal to animal. Standard deviation of mean kallikrein concentration was for instance for pilocarpine stimulated parotid saliva 33.2% and the kallikrein secretory rate varied by 46.4%. However when both glands in a pair were cannulated and exposed to the same stimulation, the saliva of the two glands differed less than among various animals (Table IV). The difference between paired glands is an indication on methodological errors in the test system.

Variations in kallikrein secretion during the stimulation period. The concentration and secretion of kallikrein in parotid saliva following parasympathetic stimulation (electrical stimulation of the auriculotemporal nerve or pilocarpine) and electrical stimulation of the sympathetic cervical nerve was found to be much higher during the first 2-3 min than during the following minutes (Fig. 1). During this initial period a large part of the parotid gland kallikrein was secreted, i.e. 0.71 ± 0.44 EU for auriculotemporal nerve stimulation ($n=5$) and

TABLE III. Concentration and secretory rate of kallikrein in parasympathetically and sympathetically induced saliva from the submandibular, the sublingual, and the parotid glands, and in urine and tears. The standard deviation for the sympathetic submandibular saliva was very high due to the large variations. The range of the kallikrein concentration and secretory rate is therefore given below in parentheses. n = number of glands investigated. When $n = 3$ the range is given in parentheses below the mean. $n = 13$ measurements were made in each of the 14 rats. The figure in the table represents the mean of the mean of each rat, thus the standard deviation indicates the biological variation between different animals. The day to day variation was found to average $14 \pm 4\%$ and $32 \pm 6\%$ for the kallikrein concentration and secretory rate respectively

	Submandibular gland				Sublingual gland				Parotid gland			
	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min	n	Stim. period min	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min 10^{-4}	n	Stim. period min	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min	n	Stim. period min
<i>Parasympathetic</i>												
Acetylcholine	137.4	1.20	1	20					58.8 (49.5-64.9)	0.095 (0.025-0.187)	3	6-20
Electrical	155.6 ± 130.9	1.76 ± 1.07^a	6	10-30	1.96 ± 0.77	19 ± 9	5	9-14	18.3 ± 11.4	0.100 ± 0.047	4	10
Pilocarpine	42.3 ± 20.3	0.31 ± 0.16	5	20-30	0.72 ± 0.37	24 ± 9	7	15	8.9 ± 3.4	0.073 ± 0.036	15	6-19
<i>Sympathetic</i>												
Cervical nerve	113.286 ± 156.720 (7.539-560.367)	1.157 ± 2.706 (0.18-9.973)	13	3-20	22.5 ± 17.0	11 ± 7	4	6-10	85.9 ± 81.9	0.098 ± 0.057	15	8-15
Noradrenaline	88.290 ± 111.522 (13.898-274.500)	201 ± 232 (15-494)	5	3-10	71.8	14	2	5-15	119.6 ± 70.6	0.121 ± 0.048	7	4-18
Isoproterenol	1.944 ± 1.669 (725-4.425)	1.3 ± 1.3^a (0.04-3.5)	6	20-40	22.1 (8.7-42.3)	16 (2.8-42.3)	3	15	42.9 ± 19.1	0.052 ± 0.017	8	15-16
Unstimulated	0.47 ± 0.05 EU/ml ($\pm 10.6\%$)	7.2 ± 2.9 EU/24 hrs ($\pm 40.3\%$)	24 hrs	$n^a = 14$								
writer:												
Parasympathetically stimulated tears, no esterase activity												

Results published in Ørstavik and Ørstavik (1977).

TABLE IV The enzyme concentration and secretory rate in saliva from the major salivary glands following various gland activation procedures. The table shows the difference between paired glands (L and R) and between different animals.

			EU/ml	Deviation from mean "	EU/ml	Deviation from mean "
Submandibular	Pilocarpine	L	34.5	10.3	0.19	22.7
		R	42.4		0.74	
	Acetylcholine	L	147.4	7.2	1.31	10.5
		R	127.3		1.08	
Parotid	Pilocarpine	L	6	3.4	0.043	0
		R	6.7		0.043	
	Pilocarpine	L	14.3	2.9	0.110	10.5
		R	13.2		0.099	
	Pilocarpine	L	8.3	6.7	0.051	24.2
		R	9.5		0.084	
	Pilocarpine	L	5.0	11.5	0.044	19..
		R	6.3		0.065	
	Isoproterenol	L	44.6	11.3	0.053	14.5
		R	36.2		0.071	
	Isoproterenol	L	70.6	7.8	0.054	22.7
		R	60.4		0.034	
	Isoproterenol	L	15.0	52.5	0.024	47.0
		R	48.2		0.067	
	Isoproterenol	L	49.5	44.0	0.067	16.5
		R	19.0		0.048	
	Acetylcholine	L	49.5	13.5	0.025	48.9
		R	64.9		0.073	
	Aminocaproic acid	L	17.2	17.5	0.083	33.7
		R	24.5		0.168	
Sublingual	Pilocarpine	L	0.57	10.9	0.0022	11.1
		R	0.71		0.0029	
	Pilocarpine	L	1.25	0.4	0.0033	4.9
		R	1.24		0.0037	
	Pilocarpine	L	0.44	8.5	0.0020	42.3
		R	0.27		0.0008	
Mean of deviation from mean				13.9	21.9	
				±14.7	±15.0	

8.91 ± 0.32 EU for cervical nerve stimulation ($n=5$). The parotid gland contained 0.82 ± 0.54 EU (a.s.).

In the submandibular gland parasympathetically induced saliva showed time-dependent decrease in kallikrein concentration and secretion (Fig. 2a and b) which was comparable to that seen in the parotid gland. Following sympathetic stimulation (Fig. 2b and c) 4 out of 5 animals showed a decrease in kallikrein concentration and secretory rate during the stimulation period. In these rats considerable amount of total gland kallikrein was secreted during the initial stimulation period. In one animal the kallikrein concentration remained unaltered. However, this particular gland produced a saliva with a kallikrein concentration in the very low range of that regularly seen on sympathetic stimulation.

Urinary kallikrein secretion. The kallikrein concentration in urine was 0.447 ± 0.048 EU/ml and the kallikrein secretory rate was 6.91 ± 2.87 EU/24 h (Table II). The day to day

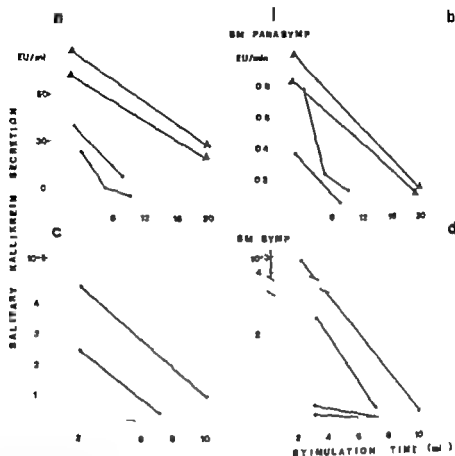


Fig. 2. Salivary kallikrein concentration (a and b) and secretory rate (c and d) during the period of parasympathetic (ductal nerve ● and pilocarpine ▲) and sympathetic (cervical nerve) stimulation of the submandibular gland.

variation for one rat was $14 \pm 4\%$ for the urinary kallikrein concentration and $32 \pm 6\%$ for the kallikrein secretory rate.

Kallikrein secretion and its relationship to salivary and urinary volume Kallikrein secretion rate was poorly correlated with salivary flow rate. Best correlation was seen after pilocarpine stimulation of the parotid gland ($r = +0.70$ $P < 0.05$). The relationship between kallikrein and salivary secretion from one gland could not be estimated, since not enough salivary samples were obtained during the stimulation period.

The day to day variations in urinary kallikrein secretory rate and concentration were compared with alterations in urine volume. It was found that individual kallikrein secretory rates were correlated with the urinary fluid volume ($r = +0.91 \pm 0.06$, $P < 0.05$). These data were based on 14 rats each providing about 15 measurements. When the mean kallikrein secretory rate for each rat was compared with the mean urine volume, the correlation was $r = +0.88$ ($P < 0.05$). No relationship was seen between urinary volume and urinary kallikrein concentration.

Kallikrein activity in tears Bz Arg-OEt-esterase activity was not detected in tears.

Discussion

In the present study quantification of glandular kallikrein was based on its esterase activity and on its antigenicity. For the esterase measurements *N*-benzoyl-L-arginine ethyl ester (Bz Arg-OEt) was used as substrate, a method which has proved to give a good estimate of kallikrein activity in rat kidney extracts and urine (Nustad 1970) as well as in rat submandibular gland extracts and saliva (Ørstavik and Gautvik 1977). Moreover quantification of kallikrein by its antigenicity has been shown to be a specific method for kallikrein measurements in rat submandibular gland extracts and saliva (Ørstavik *et al.* 1977, Ørstavik and Gautvik 1977). The enzyme test is more sensitive than the immunological method and was therefore useful for organs with low kallikrein concentration per g wet weight such as the parotid and the exorbital lacrimal glands. Since kallikrein-specific fluorescence has been detected immunohistochemically in the parotid gland (Ørstavik 1978), it is concluded that the esterase activity observed at least partially was due to kallikrein. However in the lacrimal gland neither kallikrein-specific fluorescence (Ørstavik 1978) nor esterase activity was observed. Furthermore, esterase activity could not be detected in tears obtained by stimulation of the exorbital lacrimal gland. It is therefore concluded that kallikrein is lacking or present in extremely low concentrations in this gland. In such organs as the pancreas, where kallikrein is found as a pro-enzyme, immunological kallikrein quantitations could be made (Ørstavik and Glenner 1978).

The innervation of kallikrein-containing cells can be elucidated by studying the secretory pattern of kallikrein. The large amounts of kallikrein found in the rat submandibular gland is almost entirely related to the granular tubular cells (Ørstavik *et al.* 1977) and is most probably located in the secretory granules of these cells (Ørstavik *et al.* 1975). It can thus be deduced that the granular tubules are specifically activated through α -adrenergic sympathetic receptors. The comparably very low secretory rate of kallikrein following β -adrenergic and parasympathetic stimulation indicates that the granular tubular cells are only slightly activated by such fibers. The granular tubular cells have been shown to undergo degranulation when the gland is subjected to α -adrenergic sympathetic stimulation but not following β -adrenergic or parasympathetic stimulation (Matthews 1974). These data are in agreement with the above suggested innervation pattern for the granular tubular cells.

It has been shown that pilocarpine in high doses also stimulates the superior cervical ganglion (Schroyer and Hall 1966). However since large amounts of kallikrein are secreted during cervical nerve stimulation, but not during pilocarpine stimulation, it is concluded that the doses of pilocarpine used in the present study did not activate the superior cervical ganglion.

Also in the parotid and sublingual glands the striated ducts seem to be slightly more affected by sympathetic, particularly α -adrenergic, than parasympathetic stimulation. The differences observed in kallikrein secretory rate were small and mostly not significant. However when cervical nerve stimulation was superimposed on pilocarpine stimulation, the kallikrein secretory rate from the parotid gland was shown to increase, confirming that kallikrein secretion is somewhat higher after sympathetic than after parasympathetic stimulation.

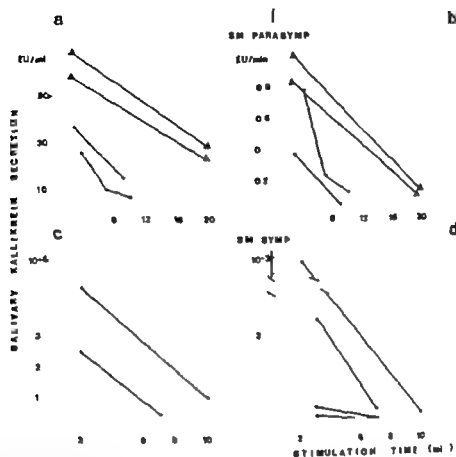


Fig. 4. Salivary kallikrein concentration (a and b) and secretory rate (c and d) during the period of parasympathetic (dual nerve ● and pilocarpine ▲) and sympathetic (cervical nerve) stimulation of the submandibular gland.

variation for one rat was 14 ± 4 % for the urinary kallikrein concentration and 32 ± 6 % for the kallikrein secretory rate.

Kallikrein secretion and its relationship to salivary and urinary volume Kallikrein secretion rate was poorly correlated with salivary flow rate. Best correlation was seen after pilocarpine stimulation of the parotid gland ($r = +0.70$ $P < 0.05$). The relationship between kallikrein and salivary secretion from one gland could not be estimated since not enough salivary samples were obtained during the stimulation period.

The day to day variations in urinary kallikrein secretory rate and concentration were compared with alterations in urine volume. It was found that individual kallikrein secretory rates were correlated with the urinary fluid volume ($r = +0.91 \pm 0.06$, $P < 0.05$). These data were based on 14 rats each providing about 15 measurements. When the mean kallikrein secretory rate for each rat was compared with the mean urine volume, the correlation was $r = +0.88$ ($P < 0.05$). No relationship was seen between urinary volume and urinary kallikrein concentration.

Kallikrein activity in tears Bz-Arg-OEt-esterase activity was not detected in tears.

The kallikrein secretory pattern found in the rat salivary glands resembles that seen in major salivary glands of other species. In the cat submandibular gland sympathetic nerve stimulation resulted in a 90–95% decrease in the total gland kallikrein content (Barton *et al.* 1975). The nerve induced kallikrein release was found to be mediated through α -adrenergic receptors (Gustvik *et al.* 1974). *In vitro* studies on the submandibular gland of the guinea pig showed that kallikrein was released on α and β -adrenergic stimulation and also, although less, on parasympathomimetic stimulation (Albano *et al.* 1976). A decrease in salivary kallikrein concentration during the stimulation period was also seen in the cat submandibular gland (Gustvik *et al.* 1974).

The large individual variation in kallikrein content in both glands and saliva and the rather small nerve induced differences observed in the parotid and sublingual salivary kallikrein, indicate that the glandular kallikrein level may be influenced by factors not controlled in the present study. Recent investigations strongly point to a role of mineralocorticoids in the secretion of urinary kallikrein (Geffer *et al.* 1972), and such hormones may also influence the secretion of salivary and other glandular kallikreins. In accordance with this concept, glandular kallikreins have been demonstrated in the kidney (Ørstavik *et al.* 1976), in the salivary glands (Ørstavik *et al.* 1975, Brandtzen *et al.* 1976, Ørstavik 1978), in the toad bladder (Chao and Margolius 1978), in sweat (Frikl *et al.* 1970), and in feces possibly in part originating from pancreatic pro-kallikrein (Ørstavik and Glenner 1978), whereas in the lacrimal gland kallikrein could not be detected. In these kallikrein-containing organs, electrolyte transport will be influenced by aldosterone (Blair West *et al.* 1967, Giebisch 1971). Moreover in the salivary glands and in the kidney the organs in which the cellular localization of kallikrein has been determined (Fig. 3), the kallikrein-containing cells are also known to be the major target cells for mineralocorticoids in these organs (Young and van Lennep 1978, Giebisch 1971).

In the present study correlation between urinary kallikrein secretion and urine volume, but not between salivary kallikrein secretion and salivary volume, was observed. A relationship between urine water and kallikrein has also been shown in previous studies (Mills and Ward 1975) but has been disputed by others (Margolius *et al.* 1974). The relationship may be due to other physiological processes taking place concomitantly with kallikrein secretion.

In conclusion, salivary kallikrein concentration is highest after sympathetic, particularly α -adrenergic, stimulation in all three major salivary glands of the rat. The kallikrein-rich parietal tubular cells of the submandibular gland are specifically activated through α -adrenergic receptors. The distribution of kallikrein in exocrine organs may be related to cells important for the regulation of the sodium/potassium balance.

I wish to thank Dr Kjell Nustad for the supply of antikallikrein serum and purified kallikreins and for the many helpful discussions.

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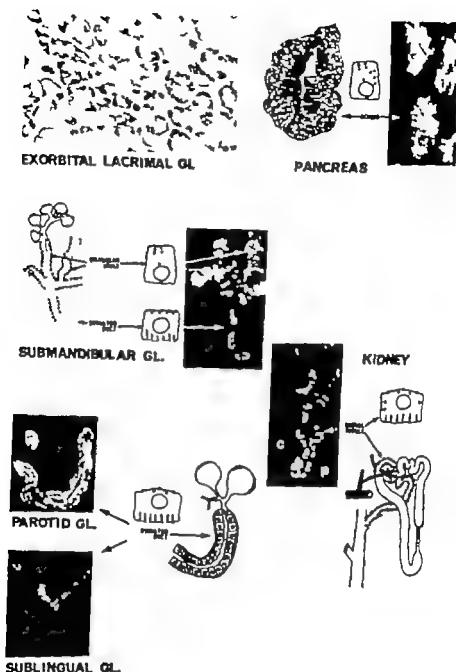


Fig. 3 Immunohistochemical demonstration of the cellular localization of kallikrein in the rat pancreas, kidney, and the submandibular, sublingual, and parotid glands. Kallikrein is in all organs, except in the pancreas, found in cells showing infoldings of the basal cell membrane associated with numerous mitochondria, illustrated by the basal striation in the line-drawings. In the pancreas, kallikrein is found as a proenzyme, probably activated in the duodenum, suggesting that pancreatic kallikrein may have an extra-glandular function. The pancreatic acinar cells resemble the granular tubular cells of the submandibular gland in morphology and by their content of trypsin-like enzymes. The exorbital lacrimal gland does not have any cells resembling the salivary striated duct cells, and kallikrein was not found in this gland.

The cellular localization of glandular kallikreins has been published previously: the pancreas in Ørstavik and Glenner (1978), the submandibular and sublingual glands in Ørstavik *et al.* (1975) and Brandtzaeg *et al.* (1976), the parotid gland in Ørstavik (1978), and the kidney in Ørstavik *et al.* (1976). Part of this figure has also been published in Nustad *et al.* (1978).

The effects of ureteral occlusion and renal venous constriction on kidney kallikrein-kinin and prostaglandin systems in dogs

By

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Abstract

OLSEN, U. B. The effects of ureteral occlusion and renal venous constriction on kidney kallikrein-kinin and prostaglandin systems in dogs. *Acta physiol. scand.* 1978. 104: 443-452.

The arterial pressure was raised to 40-50 mmHg by ureteral occlusion or by renal venous constriction in anesthetized dogs loaded with 10 mmol/l sodium and with urine flow of approximately 1 ml/min/kg. Both maneuvers produced vasodilation and decreased urine creatinine excretion (GFR). Ureteral occlusion was associated with marked antinatriuresis, which contrasted the sizable decrements in sodium excretion during renal venous constriction. Ureteral occlusion did not affect urine excretion of kallikrein or kinins, but renal venous constriction decreased urinary kallikrein excretion, and markedly increased urinary kinin excretion. Ureteral occlusion and renal venous constriction considerably increased urine prostaglandin (E-type) excretion by presumably pressure dependent mechanisms. Inhibition of prostaglandin synthesis by indomethacin abolished the vasodilation during renal venous constriction and this was accompanied by marked reductions of urinary creatinine (GFR) and kallikrein excretions, but the kinin excretion was enhanced as observed before the administration of indomethacin.

The physiological role of the kidney kallikrein-kinin system is not precisely known. Kidney kinins, kallikrein and bradykinin (Hiesl *et al.* 1976), infused into the renal artery produce vasodilation and natriuresis (Hindenreich *et al.* 1964, Webster and Gilmore 1964, Barraclough and Mills 1965, Wallis *et al.* 1969, Olsen 1978). Consequently kinins have been implicated in regulation of blood pressure and sodium balance.

Kallikrein is an enzyme which cleaves kinins from plasma substrates, kininogens. Urine kallikrein and urine kinins originate intrarenally (Yoshinaga *et al.* 1964, Nustad 1970, Nappier *et al.* 1975, Moriwaki *et al.* 1976), and are excreted in the urine at rates which probably reflect their intrarenal activity. It is generally believed, yet not proved, that urine kallikrein excretion is an index of the activity of the renal kallikrein-kinin system.

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The effects of ureteral occlusion and renal venous constriction on kidney kallikrein-kinin and prostaglandin systems in dogs

By

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Abstract

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The intravital pressure was raised 40-50 mmHg by ureteral occlusion or by renal venous constriction. Anesthetized dogs loaded with 10 µCi ¹²⁵I-saralisin in saline and with urine flow of approximately 1 ml/min/kidney. Both manoeuvres produced vasodilation and decreased urine creatinine excretion (GFR). Ureteral occlusion was associated with marked antihypertension, both counteracted the sizable decrements in sodium excretion during renal venous constriction. Ureteral occlusion did not affect urine excretion of kallikrein or kinins, whilst renal venous constriction decreased urinary kallikrein excretion, yet markedly increased urinary kinin excretion. Ureteral occlusion and renal venous constriction comparably increased urine prostaglandin (E-keto) excretion by presumably pressure dependent mechanisms. Inhibition of prostaglandin synthesis by indomethacin abolished the vasodilation during renal venous constriction and this was accompanied by marked reductions of urinary creatinine (GFR) and kallikrein excretion, whilst the kinin excretion was enhanced as observed before the administration of indomethacin.

The physiological role of the kidney kallikrein-kinin system is not precisely known. Kidney kinins, kallikrein and bradykinin (Hjal *et al.* 1976), infused into the renal artery produce vasodilation and natriorexis (Hendrenreich *et al.* 1964, Webster and Gilmore 1964, Burroughs and Mills 1965, Willis *et al.* 1969, Olsen 1978 a). Consequently kinins have been implicated in regulation of blood pressure and sodium balance.

Kallikrein is an enzyme which cleaves kinins from plasma substrates, kininogens. Urine kallikrein and urine kinins originate intrarenally (Yoshinaga *et al.* 1964, Nustad 1970, Nagler *et al.* 1973, Morawski *et al.* 1976), and are excreted in the urine at rates both probably reflect their intrarenal activity. It is generally believed, yet not proved, that urine kallikrein excretion is an index of the activity of the renal kallikrein-kinin system.

In acute experiments urinary kallikrein excretion seems related to changes in urine flow (Mills *et al.* 1976). Extreme enhancement of urine kallikrein excretion follows the administration of loop diuretics (Crouxatto *et al.* 1973, Olsen and Alhuseth Rønne 1976), while decreased urine flow during acute or chronic renal artery constriction in dogs is accompanied

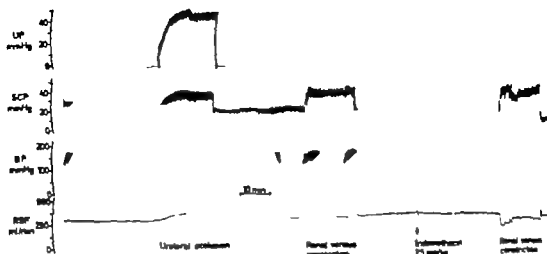


Fig. 1 Continuously registered parameters in a representative experiment. UP = ureteral pressure, SCP = subcapsular pressure, BP = arterial blood pressure and RBF = renal blood flow

by extreme reductions of urinary kallikrein excretion (Bevan *et al* 1974, Keiser *et al* 1976). Loop diuretics and renal artery constriction produce marked, yet opposite effects upon the intrarenal pressure. Consequently this has led to the hypothesis that urinary kallikrein excretion might be influenced by the intrarenal pressure (Mills *et al* 1976, Olsen and Anfelt-Ronne 1976). One of the objects of the present study was to investigate this hypothesis in anesthetized dogs, in which the intrarenal pressure was comparably raised by ureteral occlusion or by renal venous constriction. The latter procedure has previously been reported to enhance the appearance of kinins in urine (Alzamora and Capelo 1973). Therefore urine kinin excretion was also measured in the present study in order to investigate if a relationship could be demonstrated between acute changes of urine kallikrein and kinin excretions. Finally indomethacin was used as a tool to investigate the possible relationship between renal prostaglandins and the kallikrein-kinin system. The last issue was prompted by a previous report which showed that indomethacin pretreatment in dogs impaired the enhancements of urinary kallikrein excretions provoked by the administration of the loop diuretic bumetanide (Olsen and Anfelt-Ronne 1976).

Methods

6 female mongrel dogs (16–28 kg) which had been fasted overnight were used for the experiments. The dogs were kept on a standard diet (Doggy®) with free access to water.

The dogs were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and intubated for spontaneous respiration. A Statham (P23 Db) transducer was connected to carotid artery for blood pressure measurements, and a catheter was placed in the jugular vein for subsequent infusion. The abdomen was opened by midline incision. The ureters were cannulated for urine collections, and a valve was placed at the tip of the cannula in the left ureter. The valve was constructed so that the ureteral pressure could be raised to the desired pressure level of 40–50 mmHg (registered by a Statham pressure transducer) before an urine overflow was established. A flow probe was placed around the left renal artery for the measurement of renal blood flow (electromagnetic flow meter Nycotom®). A thread sling for manual constriction was placed around the left renal vein and the subcapsular (intrarenal) pressure as measured by a P19 miniature transducer (Konigberg Instr.). Blood pressure, renal blood flow and subcapsular

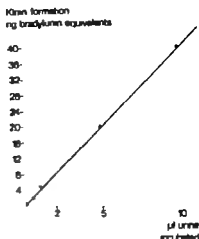


Fig. 2. Relationship between amount of urine incubated and kinin formation. Normal urine was pooled from 4 dogs. The symbols \circ and \bullet represent two separate series of incubations in which kinins have been determined on different isolated rat uterus.

pressure was continuously recorded on a polygraph. At the end of the experiment the dog was killed by lethal dose of pentobarbital. The subcapsular pressure recorded at this stage was used as zero a.s.w.

Following completion of surgery the dogs were loaded with 100 ml 10% mannitol in saline (10 ml/min) and 2 ml/min was infused throughout the experiment. Urine was collected on ice for 15 min periods. Fig. 1 shows the recordings from an experiment. When urine flow had stabilised 30–45 mmHg after surgery control urine was collected. The urine flow from the left kidney was then stopped until the arterial pressure rose to 40–50 mmHg. The initial 1–2 ml urine was discarded and subsequently 15 min urine overflow was collected at this pressure. Following 15 min of recovery control urine was again collected. The left renal vein was then constricted to raise the subcapsular pressure to the same level as that obtained during arterial occlusion, and urine was collected for 15 min. After recovery indomethacin (2 mg/kg) was administered i.v. Control urine was collected 15 min later and the procedure with constriction of the renal vein was repeated as before the administration of indomethacin. Urine collected from the right and unoccluded kidney served as an additional control to establish the effects of time and indomethacin on kidney function.

The urine samples were immediately diluted with ice cold distilled water ad 30.0 ml, in order to provide an uniform and suitable volume for subsequent extractions of prostaglandins and kinins.

During the divisions of the intrarenal pressure to 40–50 mmHg by renal vein constriction proteinuria (haematuria) was observed in 4 of the experiments, but all samples were free of blood. Haematuria was, however occasionally observed in preliminary experiments when the vein was constricted to raise the intrarenal pressure to more than 50–60 mmHg.

Analyses

Uterine kallikrein activities. Kininogenase activity in urine was determined by the microassay of Marano-Garcia and Carretero (1972), which was slightly modified. In addition to kallikrein urine contains other kininogenases, yet less active (*e.g.* plasmin, trypsin). In order to enhance the specificity of the kallikrein measurement, the heated (58°C for 3 hr) dog plasma containing kininogen was obtained from 9 ml dog whole blood collected on 1 ml aprotinin (10 000 KIU/ml Trasylol® Bayer), protease inhibitor that does not inhibit dog urine kallikrein (Morrisaki *et al.* 1976). The kinin forming incubations were then performed in 100 μl 200 μl buffer (0.1 M phosphate, pH 7.4) diluted urine (containing the urine formed in 1/100 ml) plus 100 μl Tyrode solution were incubated with 30 μl heated dog plasma in plastic tubes for 2 min at room temperature (22°C). The kinin forming reaction was stopped by placing the tubes in boiling water for 5 min, and the kinins released were subsequently assayed on the isolated rat uterus (Otter and Abelson-Rasmussen 1976). Fig. 2 shows that the kinin formation was directly proportional to the amount of urine incubated.

The incubated samples from each experiment were assayed on the same uterus with reference to tyrosine bradykinin (Kendou). Differences in kallikrein activity among samples exceeding 20% were safely detected. One unit of kallikrein is the amount of enzyme which forms 1 μg of bradykinin equivalents by the incubation procedure.

Urine prostaglandin excretion. The ice cold urine samples (30 ml) were acidified (pH 3) with 5% formic acid and lipids were extracted with an equal volume of chloroform. The water phase was frozen and stored for subsequent extraction of kinins. Polar lipids (*i.e.* prostaglandins) were purified by silicic acid chromatography (Olsen *et al.* 1976) and the biological activity was assayed on the isolated rat fundus strip in terms of PGE₁-like activity (Olsen *et al.* 1976). Recovery of ³H PGE₂ added to the urine was more than 80%. The identity of the PGE-like material was established in three of the experiments in which the samples were pooled after assay. 10 000 cpm ³H PGE₂ was added and lipids were reextracted and applied on silica coated plates. The plates were developed in the solvent system chloroform-methanol-acetic acid-water (90:9:1:0.65) and the ³H PGE₂ spot was precisely located in a Spark chamber. After zonal division the plates were scraped off and it was confirmed that the biologically active material (rat fundus strip) cochromatographed with the ³H PGE₂ standard.

Urine kinin excretion. A possible enzymatic formation of methionyl-tyr⁸-bradykinin during the acidic extraction procedures (Hjal *et al.* 1976) was avoided by the following steps. 1) Urine was maintained cold during acidification and lipid extraction and urine (water phase) was subsequently transferred to 100 µl plast flasks and stored frozen (Mitswa *et al.* 1969). 2) In 3 of the experiments the acidic protease inhibitor pepstatin (Beckman) was added (50 µg/sample) (Hjal *et al.* 1976). The results of these experiments were essentially similar to those in which pepstatin was not used. Furthermore it should be noticed that the rat uterus, which was used for the assay procedure (see below), is less sensitive to methionyl-tyr⁸-bradykinin than to bradykinin or kallidin.

Urine kinins were extracted by batch absorption to Amberlite CG-50(H) (100-200 mesh) (Mitswa *et al.* 1969; Hjal *et al.* 1976). After thawing the pH of the water phase of urine was adjusted to 4.0 and is shaken with 100 mg resin in plast flasks for 2 h. The resin was settled by standing and was transferred to plast tubes. After repeated washing with distilled water the kinins were eluted with 2 ml 9M acetic acid by shaking the resin for 1 hour. The resin was sedimented by centrifugation and the acetic acid was transferred to 3 ml tubes and was evaporated at 60°C in vacuo. Kinins were redissolved in 0.5 ml 0.9% NaCl plus 50 µl 2M Tris. Recovery of ³H-bradykinin added as internal tracer in eighteen samples from different experiments was 39 ± 3%. Approximately 10% loss of the kinins occurred during the initial extraction of prostaglandins. Kinins were assayed on the isolated rat uterus (Olsen and Ahlstedt 1976). The test samples behaved like bradykinin on the tissue and were free from interfering substances. In addition the samples produced vasodilation in the dog hind limb blood flow preparation (Marglert *et al.* 1975) and the biological activity was destroyed by carboxypeptidase B and by chymotrypsin, but not by trypsin, which in contrast enhanced the biological response of the rat uterus. These biological criteria are characteristic of kinins (Shaw and Ramwell 1968; Nargiletti *et al.* 1975). The identity was finally established utilizing ³H bradykinin as a tracer. The samples were pooled after assay and kinins were reextracted. The acetic acid eluate was concentrated and was applied on aluminiumoxid pre-coated plates (DC Alufolien, Aluminiumoxid F254 Neutral, Typ T Merck). The plates were developed in the solvent system butanol-water-acetic acid (25:12:2, the organic layer). ³H bradykinin was precisely located in the Spark chamber and appeared in one distinct spot with R_F = 0.14-0.19. Kallidin and methionyl-tyr⁸-bradykinin were run separately. R_F of kallidin was 0.06-0.09 and methionyl-tyr⁸-bradykinin mobilized in front of kallidin. After zonal division the plates were scraped off into plast tubes and kinins were eluted with Tyrodes solution. The biologically active materials (rat uterus) cochromatographed with bradykinin and kallidin zones. Other activity was absent or negligible.

Urine sodium and creatinine excretions. Sodium was determined by flame photometry and creatinine by Jaffe's reaction.

Statistics. The results are presented as mean ± S.E. The statistical significance of the results was assessed by Student's *t* test after a logarithmic transformation of paired (ratio) data.

Results

Fig. 1 shows the continuously registered parameters in a representative experiment. The elevations of the ureteral pressure to a steady level of 46 ± 1 mmHg were paralleled by similar and not significantly different elevations of the subcapsular pressure to 45 ± 3 mmHg. Table I summarizes the results of the measurements on the experimental left kidney and Table II shows the results of the control kidney.

TABLE I. The effect of ureteral occlusion and renal venous constriction on kidney parameters. Results are mean \pm S.E. (N = 6). Significance was assessed by paired comparison. $-p < 0.05$, $-p < 0.01$, $-p < 0.001$, n.s. = non-significance. \star = significance at $(1/2)^{1/2}$ level by non-parametric method. \pm below the sensitivity of the assay procedure

Parameter	Control		Ureteral occl.		Control (Recovery)		Venous constr.		Control after indometh.		Venous constr.	
	I	II	III	IV	III	IV	IV/III	IV/II	V	V/III	VI	VI/V
Renal pressure, mmHg	145 \pm 6	145 \pm 6	143 \pm 6	n.s.	146 \pm 6	n.s.	n.s.	n.s.	144 \pm 7	n.s.	146 \pm 6	n.s.
Renal blood flow, ml/min	153 \pm 39	187 \pm 30	172 \pm 43	n.s.	170 \pm 44	n.s.	n.s.	n.s.	168 \pm 47	n.s.	114 \pm 38	n.s.
Glomerular pressure, mmHg	28 \pm 3	45 \pm 3	26 \pm 4	n.s.	47 \pm 3	n.s.	n.s.	n.s.	17 \pm 2	n.s.	43 \pm 4	n.s.
Urine flow, ml/min	1.3 \pm 0.2	0.8 \pm 0.1	1.5 \pm 0.1	n.s.	1.1 \pm 0.1	n.s.	n.s.	n.s.	1.3 \pm 0.2	n.s.	0.7 \pm 0.1	n.s.
Urine creatinine, mg/min	89 \pm 23	26 \pm 8	96 \pm 27	n.s.	64 \pm 34	n.s.	n.s.	n.s.	89 \pm 39	n.s.	38 \pm 13	n.s.
Urine creatinine, μ mol/min	2.8 \pm 0.2	2.2 \pm 0.2	2.7 \pm 0.2	n.s.	2.4 \pm 0.2	n.s.	n.s.	n.s.	2.3 \pm 0.3	n.s.	1.3 \pm 0.3	n.s.
Urine kallikrein, U/min	0.7 \pm 0.2	0.7 \pm 0.2	0.7 \pm 0.2	n.s.	0.4 \pm 0.1	n.s.	n.s.	n.s.	0.7 \pm 0.2	n.s.	0.2 \pm 0.1	n.s.
Urine prostaglandin, ng/min	0.4 \pm 0.1	0.4 \pm 0.1	0.8 \pm 0.3	n.s.	5.3 \pm 2.3	\star	\star	\star	1.2 \pm 0.4	n.s.	4.1 \pm 1.6	\star
Urine PGE ₂ , ng/min	0.4	0.1	0.8 \pm 0.2	n.s.	1.1 \pm 0.3	n.s.	n.s.	n.s.				

Before indomethacin treatment

Table I and Fig. 1 show that comparable enhancements of the intrarenal pressure (subcapsular pressure) by ureteral occlusion or by renal vein constriction exhibit different hemodynamic and excretory effects. The enhanced renal blood flow and the reduced creatinine excretion (glomerular filtration rate) induced by ureteral occlusion indicate a decrease of the filtration fraction by approximately 35%. During renal venous constriction renal blood flow did not change significantly and the filtration fraction only decreased by approximately 9%.

When the intrarenal pressure was enhanced by ureteral occlusion or by renal venous constriction urine volume and sodium excretion were reduced. The percent decrease of sodium excretion generally exceeded that of creatinine excretion, and consequently fractional sodium excretions fell (Table I). This antinatriuresis was significantly more pronounced during ureteral occlusion than during renal venous constriction in which condition the changes of sodium excretion were very variable (range: 148% increase - 81% decrease).

In control urine collections (Table I and II) urine kallikrein excretion appeared to be very constant with changes less than 0.1 U/min among successive periods. Ureteral

TABLE II The effect of indomethacin and time on control kidney parameters. Results are mean \pm S.E. (N=6). Significance was assessed by paired comparison. $-p < 0.05$, n.s. = non-significance. Indomethacin (2 mg/kg) was administered I.v. at time 0.

Min	-100-85	-50-35	-15-0	0-15	15-30
Urine flow ml/min	1.2 ± 0.1	$1.4 \pm 0.2^{n.s.}$	$1.5 \pm 0.2^{n.s.}$	$1.4 \pm 0.2^{n.s.}$	1.2 ± 0.1
Sodium excretion, $\mu\text{eq}/\text{min}$	75 ± 22	$95 \pm 38^{n.s.}$	$110 \pm 43^{n.s.}$	$100 \pm 39^{n.s.}$	$67 \pm 29^{n.s.}$
Creatinine excretion, $\mu\text{mol}/\text{min}$	2.9 ± 0.3	$2.8 \pm 0.2^{n.s.}$	2.6 ± 0.3	$2.5 \pm 0.3^{n.s.}$	2.3 ± 0.3
Kallikrein excretion, U/min	0.6 ± 0.1	$0.6 \pm 0.1^{n.s.}$	$0.6 \pm 0.1^{n.s.}$	$0.6 \pm 0.1^{n.s.}$	0.6 ± 0.1
Kinin excretion, ng/min	2.2 ± 1.4	$2.1 \pm 1.4^{n.s.}$	$2.2 \pm 1.4^{n.s.}$	1.3 ± 0.3	$1.0 \pm 0.3^{n.s.}$

occlusion did not significantly affect the kallikrein excretion, which, however, was reduced in 4 of 6 expts. during renal vein constriction. The latter procedure was associated with marked yet variable enhancements of urine kinin excretion (range 0.5-13.3 ng/min) which remained unchanged during ureteral occlusion.

Renal venous constriction and ureteral occlusion similarly enhanced urinary excretion of E type prostaglandins.

In control periods it was not possible to assess any significant correlation between urine kallikrein and kinin excretions or between these parameters and other hemodynamic or excretory functions. A marked correlation ($r=0.92$, $N=6$) was assessed between sodium excretion and kinin excretion in the collections during renal venous constriction (Fig. 3).

After indomethacin treatment

The administration of indomethacin decreased the urinary excretion of prostaglandins (Table I). In addition a significant reduction of the subcapsular pressure was observed although renal blood flow and urine flow were unchanged. In one right control kidney with extremely high yet constant kinin excretion of 8.9 ng/min before the administration of indomethacin this compound produced a prompt reduction of the kinin excretion to 2.3 ng/min. Otherwise, the effects of indomethacin on the parameters were small, and it cannot

Na⁺ excretion
 $\mu\text{eq}/\text{min}$

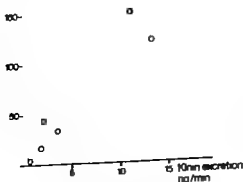


Fig. 3 Relationship between kinin excretion (abscissa) and sodium excretion (ordinate) during ureteral occlusion (●) and renal venous constriction (○).

be ruled out that they were related to time rather than to inhibition of prostaglandin synthesis.

The kinin excretion increased when the renal vein was constricted after the administration of indomethacin as also observed before the administration of indomethacin, and the urinary kallikrein excretion decreased to approximately 30% of control.

Constriction of the renal vein after inhibition of prostaglandin synthesis resulted in a marked reduction in renal blood flow (Fig. 1) and creatinine excretion (glomerular filtration rate), and a decreased urine flow and sodium excretion (Table I). Nevertheless, in 5 of 6 dogs in this condition the sodium excretion was still higher than the sodium excretion observed during ureteral occlusion before the administration of indomethacin.

Discussion

The results show that intrarenal pressure elevation by renal venous constriction increased urine kinin excretion in association with unchanged or even markedly reduced excretion of urinary kallikrein. A comparable enhancement of the intrarenal pressure by ureteral occlusion did not affect urinary kallikrein or kinin excretions. The present results therefore failed to support the hypothesis that urinary kallikrein excretion is directly related to the intrarenal pressure (see introduction).

The enhanced urinary kinin excretion during renal venous constriction observed in the present experiments agree with results previously reported by Alzamora and Capelo (1973), and probably reflects an increased intrarenal kinin formation. This can hardly be due to increased kallikrein release, since urinary kallikrein excretion was unchanged or actually decreased. More likely the change in kinin excretion could be a consequence of the increased hydrostatic capillary pressure. This might increase the microvascular surface area and produce an edema like condition with increased outward movement of fluid in association with increased permeability to proteins i.e. kininogens. At modest elevations of the intrarenal pressure by ureteral occlusion or by renal venous constriction lymph and lymph protein flow increase almost proportionally in rats (Kallakog and Wolgast 1975) and dogs (Haddy *et al.* 1959, Le Brice and Mayerson 1960, Mayerson 1963). When the venous pressure level in dogs exceeds 40 mmHg a disproportional increase of lymph protein flow has been reported, and protein concentration in lymph markedly increased (Haddy *et al.* 1959, Le Brice and Mayerson 1960). This might suggest that the availability of kininogen rather than kallikrein might be the factor which limits the rate of intrarenal kinin formation. Consequently it appears that urinary kallikrein excretion is not a reliable indicator of the intrarenal kinin activity under conditions of renal venous constriction. Neither did we observe any correlation between kallikrein and kinin excretions in the present control periods.

Indomethacin treatment did not significantly affect the control urine kallikrein excretion under the present experimental conditions. A reduction in urinary kallikrein excretion observed during renal venous constriction was, however, potentiated by the administration of indomethacin. Inhibition of prostaglandin synthesis by indomethacin in dogs has previously been reported to impair the enhancement of urinary excretions of kallikrein

which follows the cessation of renal intraarterial infusion of noradrenaline (Mills and Obika 1977) or the administration of bumetanide (Olsen and Ahnfelt-Ronne 1976). This opens up the possibility that regulation of renal kallikrein release may be influenced by local prostaglandin synthesis. The present data might suggest that kinins or the adjacent peptide fragments (Oh-lahl *et al* 1977) exhibit a negative feed-back regulation on kallikrein release, perhaps similar to that which exists between angiotensin II and renin and it is possible that prostaglandins suppress this feed-back inhibition. Alternatively the reductions of urinary kallikrein excretion during renal venous constriction after indomethacin treatment might be secondary to the marked reductions of renal blood flow and/or urine flow (Bevan *et al* 1974, Keiser *et al* 1976, Mills *et al* 1976).

Intrarenally generated kinins have been suggested to be natriuretic hormones, and rats treated with antibradykinin serum showed a reduced ability to excrete a sodium load (Marin-Grez 1974). With obvious reservations due to the low number of data the present results might support the concept that kinins exert a natriuretic function since a significant correlation between sodium and kinin excretions was observed during enhancement of the intrarenal pressure by renal venous constriction.

According to Poiseuille's law the increased outflow resistance induced by renal venous constriction should be accompanied by proportional decrements of renal blood flow all other factors being equal. The unchanged renal blood flow might therefore result from a compensatory vasodilation (Kishimoto *et al* 1972). Renal intraarterial infusion of kinins in dogs produces vasodilation and increases the appearance of prostaglandin E in blood and urine (McGiff *et al* 1972, Olsen 1978a). In the present experiments renal venous constriction and ureteral occlusion increased urinary prostaglandin excretion to a similar degree and produced renal vasodilation. An increased kinin excretion was only induced by renal venous constriction. Consequently intrarenally generated kinins cannot be the only factor responsible for the vasodilation and the prostaglandin synthesis under these conditions, if involved at all.

The increased urinary excretion of prostaglandins during ureteral occlusion or renal venous constriction is presumably mainly a result of the elevation of the intrarenal pressure. We have previously reported comparable enhancements of urinary prostaglandin excretions during similar elevations of the intrarenal pressure by ureteral occlusion and by kidney compression in anaesthetized dogs (Olsen *et al* 1976). Furthermore an intrarenal pressure dependent mechanism of prostaglandin release has been demonstrated in the isolated Krebs-Henseleit perfused rabbit kidney (Olsen 1978b). The prostaglandins released by such a pressure mechanism have been implicated in the accompanying vasodilation, since indomethacin treatment inhibited the vasodilation during ureteral occlusion or during kidney compression and caused the glomerular filtration rate to decrease severely (Olsen *et al* 1976). This was also observed in the present experiments in which indomethacin treatment impaired the vasodilation observed during renal venous constriction, and this was accompanied by decreased glomerular filtration rate (creatinine excretion). Presumably the vasodilations observed during elevation of the ureteral pressure or during renal venous constriction are mediated by prostaglandins, and both procedures have been reported similarly to redistribute the cortical blood flow toward the inner cortex (Abe *et al* 1973).

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Adenosine and cyclic AMP in cerebral cortex of rats in hypoxia, status epilepticus and hypercapnia

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Abstract

RICHTELONA, S., B. K. SJÖJÖ and E. WESTERBERG. *Adenosine and cyclic AMP in cerebral cortex of rats in hypoxia, status epilepticus and hypercapnia.* Acta physiol. scand. 1978. 104: 453-463.

Effects of hypercapnia, hypoxia and status epilepticus on cerebral cortex concentrations of adenosine, adenosine nucleotides and cyclic AMP was studied on lightly anesthetized (70% N₂O) and artificially ventilated rats. Neither hypercapnia (arterial P_{CO}₂ about 80 and about 300 mmHg) nor hypoxia (arterial P_O₂ about 15 mmHg) altered tissue concentrations of AMP, cyclic AMP or adenosine. Bicuculline-induced status epilepticus, as accompanied by increased concentrations of cyclic AMP but adenosine concentration did not change. Experiments with ischaemia, and those in which tissue hypoxia is exaggerated by unilateral carotid artery ligation, showed that tissue adenosine concentrations were elevated only when AMP concentrations rose. It is concluded that the marked increase in cerebral blood flow which occurs in hypoxia and status epilepticus is unrelated to changes in tissue adenosine concentration and that the increase in cyclic AMP does not increase hyperactivity is triggered by other mechanisms than adenosine accumulation.

Recent results suggest that adenosine may be one important factor mediating vasodilatation in the brain. The evidence is based on two main lines of results. First, at least when sufficiently pronounced, conditions associated with vasodilatation in the brain lead to dephosphorylation of AMP with accumulation of adenosine and translocation of adenosine to extracellular fluids. These conditions include electrical stimulation (Pull and McIlwain 1972, 1973; Rubio *et al.* 1975) and reduction of oxygen tension in brain secondary to hypoxia or ischaemia (Pull and McIlwain 1972; Rubio *et al.* 1975). Second, when adenosine is applied to pial vessels, dilatation occurs (Berne *et al.* 1974; Wahl and Kuschinsky 1976). In view of such results it has been suggested that release of adenosine occurs whenever there is an increase in functional activity or a restriction of oxygen supply and that adenosine mediates vasodilatation by its extracellular-intracerebral effect on resistance vessels (Rubio *et al.* 1975). Our own previous results in hypoxia and in epileptic seizures appeared inconsistent with the above hypothesis. Thus, hypoxia leads to pronounced vasodilatation without measurably affecting the tissue concentration of AMP (Bachelard *et al.* 1974; Norberg and Sjöjö 1975; Johansson and Sjöjö 1975), and, in bicuculline-induced status epilepticus, there is little or

no increase in AMP concentration in spite of marked increase in blood flow (Meldrum and Nilsson 1976, Chapman *et al* 1977). Since the 5 nucleotidase reaction, which forms adenosine from AMP seems to be mainly determined by substrate availability (Khuge *et al* 1977; increase in tissue concentration of adenosine seemed less likely.

We recently developed a sensitive chromatographic technique for measuring adenosine concentration in tissue (Nordström *et al* 1977). Results obtained showed that, in ischemia there was a strict correlation between increase in AMP and adenosine. In the present study we have examined tissue concentration of adenosine in situations accompanied by increased cerebral blood flow. Changes in adenosine concentrations were correlated to tissue concentrations of lactate and pyruvate as well as of labile phosphates, including cyclic AMP. Preliminary accounts of some of the findings have been published (Rehncrona *et al* 1977, Nilsson *et al* 1977).

Methods

Operative and sampling technique

Male Wistar rats of a S.P.F. strain were anaesthetized with 2.3% halothane, tracheotomized, immobilized with tubocurarine chloride (0.5 mg kg⁻¹), and ventilated with 70% N₂O and 30% O₂ to yield an arterial P_{CO₂} of 35–40 mmHg. After tracheotomy halothane supply was discontinued. Catheters were placed in a femoral artery for electromanometric blood pressure recording, and for anaerobic sampling of blood, and in a femoral vein for injections. Body temperature was maintained close to 37°C. In all animals, the skin over the skull bone was incised to accommodate a plastic funnel for later freezing of the brain tissue *in situ* (Poethin *et al* 1973). This technique gives a relatively slow freezing of the tissue but, since the circulation is not interrupted prior to freezing, autolytic artefacts seem to be avoided (see also Nilsson *et al* 1975).

Experimental conditions

Arterial hypoxia was induced by reducing the O₂ concentration of the insufflated gas mixture the N₂O concentration being kept constant by admixture of N₂. In some experiments, the right common carotid artery was occluded with a rubber clamp after that the cervical sympathetic trunk and the vagus nerve had been dissected free of the vessel. Status epilepticus was induced by i.c. injection of bicuculline in a dose of 1–2 mg kg⁻¹ (see Chapman *et al* 1977). In these animals, the EEG was continuously recorded. Hypercapnia was induced by adding either 10 or 40% CO₂ to the insufflated gas mixtures, with corresponding reductions in N₂O concentration.

Analytical techniques

Arterial P_{O₂}, P_{CO₂}, and pH were measured at 37°C with macroelectrodes (Eckmeyer and Co, Kiel, and Radiometer Copenhagen), with due corrections for deviations of body temperature from 37°C.

Following freezing *in situ* the brain was chiselled out at liquid nitrogen temperature and stored at -80°C until analysis. Cortical tissue from frontoparietal region was dissected, weighed and extracted with HCl-Methanol at -22°C (see Folbergrovi *et al* 1972). Further extraction (with perchloric acid) was carried out at 0°C and following centrifugation, the neutralized extracts were used for metabolite analyses. Concentrations of phosphocreatine (PCr), ATP, ADP, AMP, lactate and pyruvate were measured with the enzymatic fluorometric techniques of Lowry and Pomonneau (1972) as described in a previous communication (Folbergrovi *et al* 1972). Cyclic AMP was measured with a protein binding technique based on the methods described by Gilman (1970) and Brown *et al* (1971), using commercial kit (The Radiochemical Centre, Amersham, England). Interference with the measurements were excluded by using extraction blanks and internal standards, run together with the tissue extracts (Folbergrovi 1975). Adenosine was measured with high performance liquid chromatography (Waters, model U6K Universal Injector, 6000 A Solvent Delivery system, 440 UV Absorbance Detector) as described in a previous article (Nordström *et al* 1977). In all experimental situations, brains were analysed together with appropriate control brains. Adenosine concentrations were evaluated using several standard samples added to the extraction blanks.

Calculations

The energy charge of the adenine nucleotide pool was calculated according to Atkinson (1968). Statistical differences were evaluated with the Student's *t* test.

Results

Cerebral values

In the material published previously (Nordström *et al* 1977) control adenosine concentrations were close to $1 \mu\text{mol kg}^{-1}$. In the present study control values obtained ranged from 0.1 to $1 \mu\text{mol kg}^{-1}$. The cause of this variability is not apparent but since re-analyses of most gave values consistent with previous results the variability seemed to represent true differences in adenosine concentration. Whatever is the explanation the variability does not affect the results of the present study since experimental brains were analysed together with appropriate controls.

Cerebral ischemia

In order to assess the rate of adenosine formation in cerebral cortex during anoxia, and the maximal values obtained, rats under 70% N_2O were decapitated and tissue was frozen for analysis after 1, 5 or 15 min. In the latter two groups, the brains were kept in a water bath at 37°C before freezing. Fig. 1 shows that, during the first 5 min, adenosine concentrations rose at a rate of $50\text{--}80 \mu\text{mol kg}^{-1} \text{ min}^{-1}$. After 15 min, tissue concentrations were close to $550 \mu\text{mol kg}^{-1}$.

Hypercapnia

Results reported by Rubio *et al* (1975) indicate that there is an inverse relationship between tissue P_{CO_2} and adenosine concentration. In the present experiments, arterial P_{CO_2} was increased to either 85 or to 300 mmHg for 30 min. arterial P_{O_2} was kept at 100–140 mmHg. Mean arterial blood pressure ranged from 150–160 mmHg in all groups. As Table I shows, hypercapnia failed to alter tissue concentrations of AMP, cyclic AMP or adenosine. There was no sign of energy failure and the decrease in phosphocreatine (PCr) is explained by a pH dependent shift in the creatine phosphokinase equilibrium (see Siesjö *et al* 1972). The decrease in pyruvate and lactate could be explained by a decrease in glycolytic flux (Folbergrova *et al* 1975).

Hypoxia

In the initial series, arterial P_{O_2} was reduced to 23–25 mmHg for 1 and 2 min, respectively. The arterial P_{CO_2} was kept at 35–40 mmHg and arterial pH was between 7.35–7.45. There was no significant fall in blood pressure. Table II shows that this degree of hypoxia was associated with expected changes in lactate, pyruvate, PCr and ADP (*cf* Bachelard *et al* 1974, Norberg and Siesjö 1975). There was a slight increase in cyclic AMP after 1 min, but the supposed minor elevation of adenosine after 1 and 2 min of hypoxia was not statistically significant.

In order to further explore changes in adenosine concentration in hypoxia, three additional series of experiments were performed. In the first, arterial P_{O_2} was reduced in steps and the tissue was frozen at the end of a 15 min hypoxic period. Fig. 2 shows that, at P_{aO_2} of 35 mmHg, there was a rise in lactate concentration (and lactate/pyruvate ratio not shown) and, at P_{aO_2} 25 mmHg, there was a fall in PCr concentration as well. However, ATP/ADP

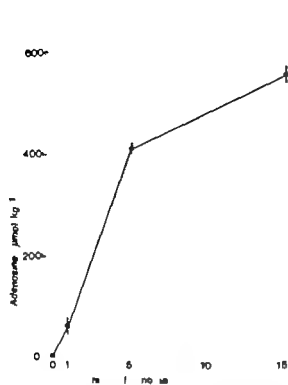


Fig. 1

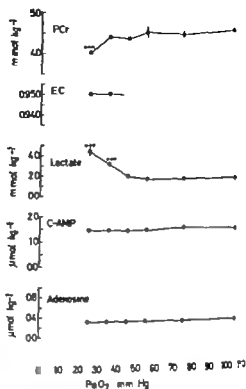


Fig. 2

Fig. 1. Rate of adenosine formation in rat cerebral cortex during anoxia. Each group consists of 3 animals, decapitated after a 30 min steady state period on 70% nitrous oxide anaesthesia with normal arterial blood gases. The brains were kept at 37°C for varying periods before freezing with liquid nitrogen. The brains of control animals (0 min of anoxia, $n = 10$: adenosine = $0.9 \pm 0.1 \mu\text{mol kg}^{-1}$) were frozen with liquid nitrogen *in situ*. Values are means \pm S.E.

Fig. 2. Concentrations of phosphocreatine (PCr), lactate, cyclic AMP and adenosine in rat cerebral cortex after 15 min at different arterial oxygen tensions ($\text{PaO}_2 = 107 \pm 3$, 73.3 ± 0.9 , 54.1 ± 0.6 , 41.6 ± 0.7 , 33.0 ± 0.5 and 26.7 ± 0.5 mmHg, respectively). Adenylate energy charge (E.C.) was calculated as $(\text{ATP} + 0.5 \text{ ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$. Each group consists of 4 animals. Values are means \pm S.E. $p < 0.001$.

and AMP (not shown), as well as the calculated adenylate energy charge remained unchanged and neither adenosine nor cyclic AMP concentrations were altered. In 3 control animals and in 5 animals subjected to a 15 min period of hypoxia ($\text{PaO}_2 \approx 25$ mmHg), cerebrospinal fluid (CSF) was sampled from cisterna magna and immediately frozen in liquid nitrogen. Mean CSF concentrations of adenosine in the hypoxic and control groups were 0.2 ± 0.1 (mean \pm S.E.), and $0.3 \pm 0.3 \mu\text{mol kg}^{-1}$ respectively. Thus the results failed to show that extracellular adenosine concentrations were elevated in hypoxia.

In the second series, arterial P_{O_2} was reduced to about 20 mmHg and the tissue was frozen after 2 min. As Table III shows, this degree of hypoxia was sufficient to alter tissue concentrations of lactate, pyruvate, PCr and ADP and there was a small reduction in adenylate energy charge (cf Norberg and Sjeström 1975). However there were no changes in concentrations of AMP, adenosine or cyclic AMP.

In the third series, arterial P_{O_2} was reduced to 20–25 mmHg in animals in which the right common carotid artery had been ligated. Table IV shows that in normoxic controls the

Table I Concentrations of phosphocreatine (PCr), adenosine nucleotides (ATP, ADP, AMP), lactate, pyruvate ($\mu\text{mol kg}^{-1}$), as well as of cyclic AMP and adenosine ($\mu\text{mol kg}^{-1}$) in rat cerebral cortex after 30 min of hypercapnia induced by ventilating the animals with 10% or 40% CO_2 .

	Control 4	10% CO_2 -4	40% CO_2 4
Pa_{O_2} kPa	41 ± 1	26 ± 5	305 ± 5
$\text{F}(\text{blood})$	7.49 ± 0.03	7.13 ± 0.02	6.68 ± 0.06
O	4.64 ± 0.10	3.94 ± 0.12	$2.36 \pm 0.07^*$
TP	3.00 ± 0.03	3.03 ± 0.02	3.05 ± 0.03
DP	0.243 ± 0.009	0.248 ± 0.004	0.260 ± 0.007
NO	0.039 ± 0.001	0.039 ± 0.001	0.043 ± 0.002
C	0.951 ± 0.001	0.951 ± 0.000	0.949 ± 0.001
lactate	1.70 ± 0.10	$0.90 \pm 0.07^*$	$1.03 \pm 0.13^{**}$
pyruvate	0.137 ± 0.009	$0.054 \pm 0.003^{**}$	0.023 ± 0.001
cAMP	1.66 ± 0.03	1.48 ± 0.11	1.62 ± 0.04
adenosine	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.1

In arterial oxygen tension was kept between 100–140 mmHg in all groups. Adenylate energy charge (E.C.) is calculated as $\text{ATP} + 0.5 \text{ADP} / (\text{ATP} + \text{ADP} + \text{AMP})$. Values are means \pm S.E. * $p < 0.05$, ** $p < 0.01$.

used also had values for labile metabolites that were close to normal (one animal was excluded since there was reduced ATP and PCr and increased ADP and AMP concentrations). In this animal, adenosine concentration was $2.6 \mu\text{mol kg}^{-1}$. In hypoxic animals, adenosine concentrations rose in 4 out of 6 animals to 17, 104, 107 and $140 \mu\text{mol kg}^{-1}$ respectively; however, this increase was correlated to a derangement of cerebral energy state with markedly increased AMP concentrations. Interestingly enough, cyclic AMP concentrations were increased (above $\mu\text{mol g}^{-1}$) only in those three animals in which adenosine concentrations rose above $100 \mu\text{mol kg}^{-1}$.

Table II Concentrations of phosphocreatine (PCr), adenosine nucleotides (ATP, ADP, AMP), lactate, pyruvate ($\mu\text{mol kg}^{-1}$), the calculated adenylate energy charge (E.C.), and lactate/pyruvate (La/Py) ratio, as well as the concentrations of cyclic AMP and adenosine ($\mu\text{mol kg}^{-1}$) in rat cerebral cortex after short periods (1 and 2 min) of hypoxia.

	Control (n = 4)	Hypoxia 1 min (n = 4)	Hypoxia 2 min (n = 4)
Pa_{O_2}	134 \pm 4.7	22.5 \pm 1.6	23.2 \pm 1.0
PCr			
ATP	4.51 \pm 0.10	3.70 \pm 0.15	3.87 \pm 0.14**
ADP	2.97 \pm 0.04	2.93 \pm 0.03	2.93 \pm 0.02
AMP	0.278 \pm 0.002	0.312 \pm 0.006**	0.291 \pm 0.004*
E.C.	0.038 \pm 0.001	0.037 \pm 0.001	0.038 \pm 0.002
Lactate	0.944 \pm 0.000	0.941 \pm 0.001	0.944 \pm 0.000*
Pyruvate	1.58 \pm 0.06	1.05 \pm 0.19*	3.21 \pm 0.20***
La/Py	0.116 \pm 0.008	0.156 \pm 0.005	0.168 \pm 0.007*
cAMP	13.7 \pm 0.67	19.6 \pm 1.28*	19.1 \pm 0.57*
Adenosine	1.34 \pm 0.03	1.50 \pm 0.06	1.43 \pm 0.06
	1.1 \pm 0.1	1.3 \pm 0.2	1.5 \pm 0.1

Values are means \pm S.E. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE III Concentrations of phosphocreatine (PCr), adenine nucleotides (ATP ADP AMP), lactate/pyruvate ($\mu\text{mol kg}^{-1}$) the calculated adenylate energy charge (E.C.) and lactate/pyruvate ratio as well as the concentrations of cyclic AMP and adenosine ($\mu\text{mol kg}^{-1}$) in rat cerebral cortex after min of pronounced arterial hypoxia ($\text{PaO}_2 < 20 \text{ mmHg}$).

	Control (n = 6)	Pronounced hypoxia 2 min (n = 6)
PaO_2	116 ± 3.8	19.0 ± 0.3
PCr	4.42 ± 0.06	$3.39 \pm 0.03^*$
ATP	3.00 ± 0.02	3.00 ± 0.01
ADP	0.285 ± 0.004	0.305 ± 0.002
AMP	0.049 ± 0.001	0.047 ± 0.001
E.C.	0.943 ± 0.001	0.940 ± 0.000
Lactate	1.60 ± 0.07	$5.40 \pm 0.20^*$
Pyruvate	0.123 ± 0.004	$0.225 \pm 0.007^*$
La/Py	13.0 ± 0.4	$24.0 \pm 0.7^*$
C-AMP	1.46 ± 0.03	1.45 ± 0.04
Adenosine	0.4 ± 0.0	0.4 ± 0.1

* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$

In conclusion, results obtained in hypoxia demonstrate that adenosine concentrations cerebral cortex are elevated above normal only when the tissue hypoxia is sufficiently pronounced to elevate AMP concentrations.

Status epilepticus

Tissue was frozen *in situ* either 10 s or 20 min following induction of status epilepticus with bicuculline. In the first group the first spike discharges appeared in the EEG 7.5 ± 0.6 s following injection of bicuculline, and freezing was thus started about 2–3 s after the beginning of seizure discharge. In the second group animals showed a continuous seizure discharge during the entire 20 min period. Mean arterial blood pressure ranged from 140–170 mmHg.

TABLE IV The influence of 30 min of hypoxia with restricted hyperemia (hypoxia combined with ipsilateral carotid artery occlusion) on the cortical energy state as well as on the cortical concentrations of adenosine and cyclic AMP in the ipsilateral hemisphere of 6 individual rats. The values (mean \pm S.E.) in the control group represents the concentrations in the ipsilateral hemisphere of 3 rats subjected to unilateral carotid artery occlusion + normoxia during 30 min. Adenylate energy charge was calculated as $[\text{ATP}] + 0.5[\text{ADP}]/[\text{ATP}] + [\text{ADP}] + [\text{AMP}]$.

	PaO_2 mmHg	PCr $\mu\text{mol kg}^{-1}$	ATP	ADP	AMP	E.C.	Adenosine	C-AMP
							$\mu\text{mol kg}^{-1}$	
Controls (n = 5)	119 ± 6	4.47 ± 0.10	3.06 ± 0.03	0.299 ± 0.007	0.035 ± 0.002	0.948 ± 0.002	0.9 ± 0.1	1.61 ± 0.09
	22.8	2.63	3.04	0.369	0.053	0.931	0.7	1.72
	19.4	3.95	3.08	0.322	0.041	0.941	1.0	1.47
Hypoxia (individual values)	21.6	0.93	2.41	0.809	0.273	0.806	18.6	1.64
	22.1	0.68	1.80	1.12	0.362	0.719	104	4.50
	21.0	1.62	1.42	1.36	0.801	0.586	107	2.59
	24.9	0.19	0.52	1.55	1.40	0.373	140	2.40

TABLE V Cortical concentrations of phosphocreatine (PCr), adenine nucleotides (ATP, ADP, AMP), lactate, pyruvate ($\mu\text{mol kg}^{-1}$), the calculated adenylate energy charge (E.C.), the sum of the adenine nucleotides (ΔAd), and the lactate/pyruvate ratio, as well as concentrations of cyclic AMP and adenosine ($\mu\text{mol kg}^{-1}$) in rats subjected to seizures induced by bicuculline. The brains were frozen *in vivo* with liquid nitrogen 2-3 s after the first spike discharges appeared in the EEG (see text) or after 20 min period of continuous seizure activity

	Control 10	Time after bicuculline inj.	
		III -4	20 min -6
PCr	4.54 ± 0.06	$2.60 \pm 0.07^{***}$	3.07 ± 0.06
ATP	2.93 ± 0.02	$2.79 \pm 0.03^{***}$	$2.76 \pm 0.01^{***}$
ADP	0.273 ± 0.012	0.317 ± 0.007	0.279 ± 0.004
AMP	0.040 ± 0.001	$0.047 \pm 0.003^*$	0.043 ± 0.001
E.C.	0.946 ± 0.002	$0.933 \pm 0.001^{**}$	0.940 ± 0.001
ΔAd	3.90 ± 0.02	$3.16 \pm 0.02^{**}$	$3.09 \pm 0.01^{***}$
La	1.45 ± 0.05	3.53 ± 0.04	$11.6 \pm 0.07^*$
Py	0.126 ± 0.006	$0.240 \pm 0.017^{***}$	0.261 ± 0.006
La, Py	13.2 ± 0.4	14.7 ± 0.5	$44.4 \pm 0.9^*$
cAMP	1.51 ± 0.03	$2.80 \pm 0.19^{**}$	$2.25 \pm 0.17^*$
Adenosine	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.0

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

control as well as in seizure groups and arterial P_{O_2} was kept above 100 mmHg. In the control group ($n = 10$) arterial P_{O_2} was 41.1 ± 0.7 mmHg, and pH was 7.39 ± 0.1 . The values fell to 23.3 ± 1.1 mmHg and 7.18 ± 0.02 , respectively after 20 min of seizure activity ($n = 6$).

Table V shows that there was a fall in phosphocreatine (PCr) and ATP with small reductions in adenylate energy charge and adenine nucleotide pool in both seizure groups. After 20 min of seizure activity there was a marked increase in tissue lactate concentration, as well as of the lactate/pyruvate ratio. In both groups there were highly significant increases in cyclic AMP but unchanged adenosine concentrations.

During the seizure periods a very small, but statistically significant increase in AMP concentration (between 0.005 and 0.007 $\mu\text{mol kg}^{-1}$) was found, which corroborate the results reported by Chapman *et al.* (1977). This very small increase in AMP did not influence the measured adenosine levels, indicating that AMP is a more sensitive indicator of changes in the phosphorylation state than adenosine. According to the regression line (adenosine ($\mu\text{mol kg}^{-1}$) = 0.06 AMP ($\mu\text{mol kg}^{-1}$) + 1.7) describing the relationship between AMP and adenosine concentrations in shortlasting ischemia (Nordström *et al.* 1977), the increase in AMP concentrations in the seizure groups, was probably too small to measurably affect the adenosine concentration.

Discussion

The present study has given three main results. First, the maximal rate of adenosine formation in ischemia is about $1 \mu\text{mol kg}^{-1} \text{ s}^{-1}$ which is somewhat higher than the rate reported by Deuticke *et al.* (1966). Second, results obtained in hypercapnia give no indication that increased oxygen tensions reduce adenosine concentrations below normocapnic control values. Third, neither hypoxia nor epileptic seizures are accompanied by elevated adenosine

TABLE III. Concentrations of phosphocreatine (PCr), adenine nucleotides (ATP, ADP, AMP), lactate, pyruvate (mmol kg^{-1}), the calculated adenylate energy charge (E.C.) and lactate/pyruvate ratio, as well as the concentrations of cyclic AMP and adenosine ($\mu\text{mol kg}^{-1}$) in rat cerebral cortex after 2 min of pronounced arterial hypoxia ($\text{Pao}_2 \approx 20 \text{ mmHg}$).

	Control (n = 6)	Pronounced hypoxia 2 min (n = 6)
Pao_2	116 ± 3.8	19.0 ± 0.3
PCr	4.42 ± 0.06	3.39 ± 0.03
ATP	3.00 ± 0.02	3.00 ± 0.01
ADP	0.283 ± 0.004	$0.303 \pm 0.002^*$
AMP	0.049 ± 0.001	0.047 ± 0.001
E.C.	0.943 ± 0.001	$0.940 \pm 0.000^*$
Lactate	1.60 ± 0.07	$3.40 \pm 0.20^*$
Pyruvate	0.123 ± 0.004	$0.225 \pm 0.007^*$
La/Py	13.0 ± 0.4	$24.0 \pm 0.7^*$
C-AMP	1.46 ± 0.03	1.45 ± 0.04
Adenosine	0.4 ± 0.0	0.4 ± 0.1

$^*p < 0.05$ $^*p < 0.01$ $^*p < 0.001$

In conclusion results obtained in hypoxia demonstrate that adenosine concentrations in cerebral cortex are elevated above normal only when the tissue hypoxia is sufficiently pronounced to elevate AMP concentrations.

Status epilepticus

Tissue was frozen *in situ* either 10 s or 20 min following induction of status epilepticus with bicuculline. In the first group, the first spike discharges appeared in the EEG 7.5 ± 0.6 s following injection of bicuculline, and freezing was thus started about 2–3 s after the beginning of seizure discharge. In the second group, animals showed a continuous seizure discharge during the entire 20 min period. Mean arterial blood pressure ranged from 140–170 mmHg in

TABLE IV. The influence of 30 min of hypoxia with restricted hyperemia (hypoxia combined with unilateral carotid artery occlusion) on the cortical energy state, as well as on the cortical concentrations of adenosine and cyclic AMP in the ipsilateral hemisphere of 6 individual rats. The values (means \pm S.E.) in the control group represents the concentrations in the ipsilateral hemisphere of 3 rats subjected to unilateral carotid artery occlusion at normoxia during 30 min. Adenylate energy charge was calculated as $[\text{ATP}] + 0.5[\text{ADP}]/[\text{ATP}] + [\text{ADP}] + [\text{AMP}]$

	Pao_2 mmHg	PCr mmol kg^{-1}	ATP	ADP	AMP	E.C.	Adenosine $\mu\text{mol kg}^{-1}$	C-AMP
Controls (n = 5)	119 ± 6	4.47 ± 0.10	3.06 ± 0.03	0.299 ± 0.007	0.035 ± 0.002	0.948 ± 0.002	0.9 ± 0.1	1.61 ± 0.09
	22.8	2.63	3.04	0.369	0.053	0.931	0.7	1.72
	19.4	3.93	3.08	0.322	0.041	0.941	1.0	1.47
	21.6	0.93	2.41	0.809	0.273	0.806	16.6	1.64
Hypoxia (individual values)	22.1	0.68	1.80	1.12	0.362	0.719	104	4.50
	21.0	1.62	1.42	1.36	0.801	0.586	107	2.59
	24.9	0.19	0.52	1.55	1.40	0.373	140	2.40

hypothesis they do not exclude the possibility that adenosine is an important regulator of cerebrovascular resistance. Thus, since the true intra- and extracellular concentrations are close to the limits of detection moderate increases in concentration, that may well have vascular effects, may be difficult to detect. Furthermore, if adenosine is released at extracellular sites by mechanisms akin to those postulated for purinergic nerves (see Burnstock 1975), and if adenosine is removed by cellular uptake mechanisms, overall tissue concentrations might not rise.

In summary we conclude that if adenosine mediates vasodilatation in the brain it must do so via more subtle mechanisms than those involving increased breakdown of AMP triggered by an imbalance between energy conserving and energy utilization pathways, with a consequent accumulation of adenosine in the brain.

Relationship between adenosine and cyclic AMP Our previous results demonstrated a linear relationship between tissue concentrations of adenosine and cyclic AMP during short-lasting ischemia (Nordström *et al.* 1977). In the present work we found no increase in the tissue levels of cyclic AMP (or adenosine) in hypoxia, if the increase in CBF was sufficient to provide the tissue with oxygen to keep a normal energy balance and prevent a deterioration of the phosphorylation state of the adenine nucleotides. Only if hypoxia was exaggerated with unilateral carotid artery occlusion (hypoxia with restricted hyperemia) was there an increase in cyclic AMP concentration, provided that the tissue hypoxia had been severe enough to cause a derangement of the phosphorylation state with pronounced accumulation of adenosine. Thus, in situations with marked disturbances of the cerebral energy state, adenosine accumulation may well trigger an increase in cyclic AMP concentration.

Earlier investigations have demonstrated an increase in cyclic AMP during neuronal hyperactivity. This has been observed in electrically stimulated brain slices (Kakuchi *et al.* 1969) as well as *in vivo* after pharmacologically or electrically induced seizures (Sattin 1971). Furthermore, *in vitro* experiments have demonstrated increased concentration of cyclic AMP in perfused brain slices after incubation with adenosine (Sattin and Rall 1970; Schultz and Daly 1973). However the present results with bicuculline-induced seizures give evidence of a highly significant increase in cyclic AMP without any change in adenosine concentration (or substantial change in phosphorylation state). We thus conclude that other mechanisms than adenosine accumulation seem to be responsible for the increase in tissue concentration of cyclic AMP during neuronal hyperactivity.

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concentrations unless there is accumulation of AMP. Since the results are in variance with data published by others we will discuss these results, and put some emphasis on the possible involvement of adenosine in the control of CBF as well as on its relationship to cyclic AMP concentration. However it seems appropriate to begin by considering tissue adenosine concentration under resting control conditions.

Cortical adenosine concentration under control conditions With conventional chromatographic techniques, adenosine concentrations in cerebral tissues are below the limit of detection (e.g. Deuticke *et al.* 1966). More sensitive techniques were used by Kleihues *et al.* (1974) in cats, by Berne *et al.* (1974) in dogs, and by Rubio *et al.* (1975) in rats who obtained values of about 20, 10 and 5 $\mu\text{mol kg}^{-1}$ respectively. However in these studies the tissue was removed with a spatula or a bone rongeur inducing shortlasting ischemia before freezing occurred. Our previous results (Nordström *et al.* 1977) as well as the present ones, have shown that if the tissue is frozen through the exposed dura or the exposed bone without prior interruption of the circulation, adenosine concentrations do not exceed 1 $\mu\text{mol kg}^{-1}$. Our previous results also showed that, at least in rats, the moderate trauma induced by a craniotomy may lead to markedly elevated adenosine concentrations, and values of 1 $\mu\text{mol kg}^{-1}$ or lower are obtained only if special precautions are taken not to damage the tissue during the craniotomy (see Nordström *et al.* 1977). We conclude that measurements of tissue adenosine concentrations require optimal freezing conditions. Since the present technique gives high values for PCr and ATP and low ones for ADP and AMP we conclude that the freezing conditions should be relatively optimal for adenosine as well.

Relationship between tissue P_{O_2} and adenosine concentration Results reported by Rubio *et al.* (1975) suggested that there was an inverse relationship between tissue P_{O_2} and adenosine concentration since the latter was reduced in hypercapnia and increased in moderate hypoxia (10–0%). Our data fail to corroborate these results since adenosine concentrations were unaltered in hypercapnia and in hypoxia, whether moderate or severe. In these situations, tissue AMP concentrations were unaltered and the unchanged adenosine concentrations thus supports the assumption that the 5'-nucleotidase reaction is substrate-dependent (Kluge *et al.* 1972). Additional support for this view was obtained by results in hypoxic animals in which the tissue hypoxia was exaggerated by unilateral carotid artery ligation (cf. Salford *et al.* 1973; Salford and Siesjö 1974). Thus, adenosine concentrations rose only when hypoxia was sufficiently severe to raise AMP concentrations above normal.

Coupling of adenosine concentration and CBF Using the same experimental conditions we have previously measured CBF in hypoxia and status epilepticus, and these measurements have been correlated to tissue concentrations of labile organic phosphates (for literature, see Jöhanisson and Siesjö 1975; Siesjö *et al.* 1975; Nilsson *et al.* 1977). These results demonstrate that the present degrees of hypoxia increase CBF about 5-fold without altering tissue AMP concentrations. In status epilepticus, CBF increases by similar degrees, and there is either a very small, or no increase in AMP concentration (Meldrum and Nilsson 1976; Chapman *et al.* 1977). Obviously the data give no support to the hypothesis that vasodilatation in hypoxia or during epileptic seizures is caused by accumulation of adenosine in the tissue.

It should be explicitly stated that although the present results fail to support the adenosine

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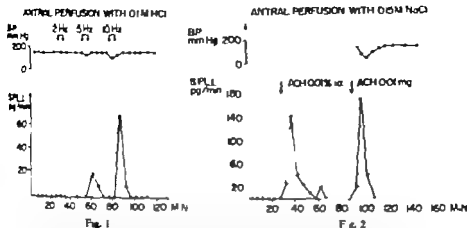


Fig. 1

Fig. 2

Fig. 1. Release of SP-LI into the antral lumen following electrical vagal stimulation with 2, 5 and 10 Hz. In the experiment no release was induced by vagal stimulation with 2 Hz, whereas stimulation with 5 and 10 Hz did cause such release. In this experiment the antrum was perfused with 0.1 M HCl.

Fig. 2. Release of SP-LI into the antral lumen following perfusion of the antrum with 0.01 M Ach solution and following injection of 1 mg Ach intravenously. The antrum was perfused with 0.15 M NaCl.

acid periods in tubes surrounded by ice. Acid samples were neutralized with 0.01 M NaOH, using phenolphthalein as an indicator. Then all samples were boiled for 10 min and stored at -20°C until assayed for SP-LI content by radioimmunoassay. The details of this assay have been given in previous papers (Nilsson *et al.* 1973, Nilsson *et al.* 1977). The antibodies used (K 25) are directed against the whole SP molecule. Removal of one single amino acid from the SP molecule virtually abolishes all immunoreactivity with this antibody (T. *in press*).

The vagi were isolated and ligated in the cervical region. The distal parts of the nerves were placed on electrodes which were connected to Grass stimulator (S 88). Stimulation was performed at the following parameters: 10 V, 2 sec duration and 2–10 Hz.

To test the effect of acetylcholine (Ach), this substance was added to the perfusion solutions (HCl and NaCl) at concentrations ranging from 0.01–1% and the antrum was perfused for 10 min. In 2 experiments 900 µg Ach was injected i.v. Adrenaline (0.01 mg) was also injected in 3 experiments.

The release rate of SP-LI (pg/min) was calculated by multiplying SP-LI concentrations and the perfusion rate. In order to obtain the amount of SP-LI released by the different stimulatory agents, basal release was subtracted from the total output of SP-LI observed after stimulation.

Results

SP-LI was found in antral perfusates in 8 of 12 investigated cats. The basal release varied between 0 and 60 pg/min. pH of the perfusion medium did not seem to influence the release rate.

Electrical vagal stimulation caused a release of SP-LI into the antrum (Fig. 1). Release responses (100–800 pg) were obtained within a frequency range from 2–10 Hz. The output varied between 100–500 pg during perfusion with NaCl and between 170–540 pg during perfusion with HCl (Table I).

Antral perfusion with Ach (0.01–1%) caused a release of SP-LI (400–1700 pg) as did i.v. injections (0.01 mg) (Table II, Fig. 2). Adrenaline (0.01 mg) i.v. released SP-LI as well (Fig. 3 Table II).

Release of substance P like immunoreactivity into the antral lumen of cats

By

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Abstract

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SPLI can be detected in antral perfusates of cats. Experiments were performed in anesthetized cats provided with antral pouches. The pouches were perfused with 0.1 M HCl and 0.15 M NaCl. The concentration of SPLI in the perfusates was determined by radioimmunoassay. Basal output varied between 0-60 pg/min. Electrical vagal stimulation (2-10 Hz) caused release responses varying between 100-800 pg. Ach given i.v. or intraantrally also released SPLI into the antral lumen as well as adrenaline given i.v.

The central nervous system and the intestine, wherefrom substance P (SP) was originally extracted (Euler and Gaddum 1931) constitute the two quantitatively most important locations of SP in the mammalian body (Pernow 1953). In the gastrointestinal tract substance P-like immunoreactivity (SPLI) has been demonstrated in nerves as well as in endocrine like cells (Nilsson *et al.* 1975, Pearse and Polak 1975).

Other gastrointestinal peptides are released into the gastrointestinal lumen in parallel to being secreted into the blood stream. Thus gastrin, somatostatin and VIP have been demonstrated in antral and/or duodenal perfusates following electrical vagal stimulation (Uvnäs-Wallensten and Rehfeld 1976, Uvnäs-Wallensten *et al.* 1977, Uvnäs-Wallensten 1978, Uvnäs-Wallensten *et al.* 1978 a, b). We therefore looked for SPLI in antral perfusates.

Methods

The experiments were performed on 12 cats (2-4 kg) fasted for 18 h and anesthetized with chloralose-urethane (50 and 100 mg/kg i.v.). The blood pressure was recorded through a catheter inserted into one of the femoral arteries. The rectal temperature was recorded and kept at 38-39°C by heating the animal with an electrical heating pad. In all cats an acute antral pouch was created by tying ligatures around the pylorus and the antrum-corpus boundary care being taken to avoid damage to nerves and vessels. The pouch was perfused with 0.15 M NaCl or 0.1 M HCl introduced via a pyloric catheter at a rate of 1.0 ml/min. The perfusate left the antrum through a cannula inserted into the antral wall and was collected during 5 or 10

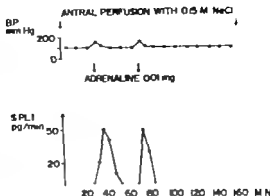


Fig. 3 Release of SPLI into the antral lumen following injections of adrenaline (0.01 mg) to the antrum as perfused with 0.15 M NaCl.

modulator actions (Euler and Gaddum 1931 Pernow 1953 Hedqvist and Euler 1975). The present results show that antral SPLI can be released by nervous activation (electrical vagal stimulation) as well as by humoral agents (Ach and adrenaline). Whether the SPLI release caused by these mechanisms are of any physiological importance cannot be stated at present. Other gastrointestinal peptides such as gastrin, somatostatin and VIP can also be released by electrical vagal stimulation within the same frequency range (2–10 Hz) (Uvnäs-Wallensten *et al.* 1976, Uvnäs-Wallensten *et al.* 1977 Schaffalitsky de Muckadell *et al.* 1977). This similarity indicates that the release of SPLI caused by vagal stimulation may occur physiologically just as the well known vagally induced gastrin release.

In conclusion we have shown that SPLI occurs in antral perfusates of cats. We have also been able to induce a release of SPLI by electrical vagal stimulation, by Ach given intraluminally or intravenously and also by adrenaline given i.v.

The study was supported by grants from Wibergs Stiftelse, Statens Medicinska forskningsråd and Bergvalls stiftelse.

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TABLE I Release of SPLI into the antral lumen of cats induced by electrical vagal stimulation (2-10 Hz) during antral perfusion with 0.15 M NaCl and 0.1 M HCl.

Exp. no.	Hz	SPLI pg	
		0.15 M NaCl	0.1 M HCl
1	2		0
2	5		170
3	10		550
4	10		210
5	2	100	
6	5	255	
7	5	800	
8	5	180	
9	10	190	

Discussion

The present results thus show that SPLI like gastrin, somatostatin and VIP is released into the antral lumen of cats. An increased output of SPLI could be induced by electrical vagal stimulation, by Ach given i.v. or into the antrum as well as by adrenaline given i.v.

Recent in vitro studies have shown SPLI to be released from nervous tissue in response to electrical stimulation and to elevation of potassium levels in the surrounding medium (Otsuka *et al.* 1976, Iversen *et al.* 1976).

SPLI occurs in nervous structures as well as in endocrine cells within the gastrointestinal tract. No SPLI containing cells have been observed proximal to the pylorus. If so the released peptide must be assumed to diffuse through the mucosal layer to reach the lumen.

The physiological function of SP in the gastrointestinal tract is still unsolved. SP has by several authors been given a hypothetical role as a regulator of gut motility but SP also has

TABLE II Release of SPLI (pg) induced by 0.01, 0.1 and 1% solutions of Ach administered via the perfusate and by intravenous injections of Ach (0.01 mg) and 0.01 mg of adrenaline given i.v. The release of SPLI was calculated as concentration of SPLI in perfusate times flow rate (l/min). The output caused by a stimulation was calculated by subtracting basal release of SPLI from total amounts observed after the stimulation.

Exp. no.	Ach %	SPLI pg	
		0.15 M NaCl	0.1 M HCl
1	0.01		938
2	0.1		650
3	1		1700
4	1	1100	
5	0.01 mg (i.v.)	850	
6			400
	Adrenaline mg		
1	0.01	500	
2	0.01	400	
3	0.01		100

Inhibition of acetylcholine release in guinea pig ileum by adenosine

By

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Abstract

GUSTAFSSON, L., P. HEDQVIST, B. B. FREDHOLM and G. LUNDQVIST. *Inhibition of acetylcholine release in guinea pig ileum by adenosine*. Acta physiol. scand. 1978. 104. 469-478.

The effect of adenosine on cholinergic neuroeffector transmission was studied in the isolated guinea pig ileum. Adenosine caused dose-dependent and inverse frequency-dependent inhibition of contraction responses to transmitter nerve stimulation. Blockade of adrenergic neurotransmission did not alter the inhibitory effect of adenosine. Adenosine also inhibited contraction responses to serotonin, angiotensin and high potassium, but not the responses to acetylcholine, histamine or direct electrical stimulation of the smooth muscle cells. Adenosine had little effect on basal outflow of acetylcholine but inhibited markedly and reversibly the release of acetylcholine induced by nerve stimulation. Acetylcholine was determined with gas chromatography-mass spectrometry. The results provide direct evidence that adenosine inhibits cholinergic neuroeffector transmission in the gut by prejunctional action on acetylcholine release. This may be of functional importance since adenosine compounds are released during stimulation of visceral nerves.

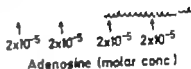
Key words: acetylcholine, adenosine, choline, guinea pig ileum, gas chromatography-mass spectrometry, neuroeffector transmission, smooth muscle, transmitter release.

Modulation of transmitter release from nerve endings by substances released from nerves (e.g. the transmitter itself) or from the effector organ has been demonstrated especially as regards sympathetic nerves (cf. Starke 1977, Westfall 1977). Recently adenosine was proposed as a possible modulator of adrenergic neuroeffector transmission in the kidney adipose tissue and vas deferens (Hedqvist and Fredholm 1976). There is evidence in the literature that adenosine (and other purine compounds) also affect acetylcholine (ACh) release from motor nerve endings (Ginsborg and Hirst 1972, Ribeiro and Walker 1975) as well as from cholinergic nerves in the gut (Sawynok and Jhaerndes 1976, Vizi and Knoll 1976, Leighton and Parmeter 1977). It therefore seemed of interest to further study if adenosine could interact with cholinergic autonomic neurotransmission, and to study this in a system where it would be possible to directly measure release of the putative neurotransmitter substance. The plexus-containing longitudinal muscle preparation of guinea pig ileum has offered a useful system to elucidate the adrenergic interaction with ACh release (Paton and Vizi 1969, Kosterlitz *et al.* 1970, Wikberg 1977) and was therefore chosen for the present study on adenosine interaction with cholinergic nerves. A preliminary account of the present results has been presented elsewhere (Gustafsson *et al.* 1977).

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Naloxone 10^{-6} M

Fig. 1 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Wash at dot. Adenosine added to bath fluid during the last half of experiment as indicated by horizontal bar.



Results

The isolated longitudinal muscle of the guinea pig ileum contracts in response to transmural stimulation, given as single pulses or trains of pulses of 1 ms duration. Since the response is abolished by atropine ($3 \cdot 10^{-9}$ M) or tetrodotoxin ($3\text{--}6 \cdot 10^{-8}$ M) it is probably due to activation of cholinergic nerve fibres. The contraction response to this type of stimulation was inhibited by adenosine administered over a wide range of concentrations. The inhibition was noticed after 10–20 seconds, usually reached its maximum within 1–2 minutes and was easily reversed upon washing (Fig. 1). The inhibitory effect of adenosine was essentially

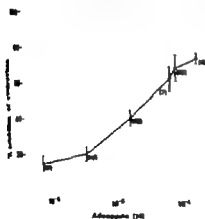


Fig. 2

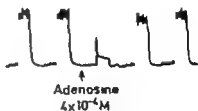


Fig. 3

Fig. 2 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Vertical axis: The depression by adenosine of contraction responses expressed as percent of the response prior to the administration of drug. Horizontal axis: Log molar concentration of adenosine. Mean values, number of experiments in brackets. Vertical bars indicate \pm S.E.

Fig. 3 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 540 pulses, 1 ms, supramaximal voltage) 9 min intervals. Wash at dot.

Methods

General procedure

Male guinea pigs (300–300 g) were stunned and bled. The distal part of the ileum was isolated and was used for two types of preparations (I) plexus-containing longitudinal muscle according to Paton and Vin (1969), and (II) whole segment of ileum with intact perivascular nerves as described by Finlén (1930). The preparation was mounted in organ baths containing Tyrode a solution of the following composition (concentrations in mM): Na 149, K 2.7, Ca 1.8, Mg 0.5, Cl 144, HCO_3^- 11.9, H_2PO_4^- 0.4, glucose 5.5. The Tyrode was kept at 37°C and was continuously bubbled with 5% CO_2 in O_2 .

Effector responses in longitudinal muscle

The preparation (20–100 mg) was mounted in a 5 ml bath. Muscle tone and contractions were recorded isotonically (load 0.2–0.5 g) using a smooth muscle transducer (Harvard Apparatus 343) and an ordinate writer (Honeywell Electronik 194). Biphasic single pulses or trains of pulses (duration 1 ms, supramaximal voltage), specifically activating the nerves, were delivered by means of a pair of platinum electrodes along the walls of the bath, and a Grass S88 stimulator. In experiments where tetrodotoxin was used the bath volume was 125 ml and the electrodes were placed 5 mm from and parallel to the preparation. Direct stimulation of the smooth muscle cells was accomplished with trains of square wave pulses (duration 20–30 ms, supramaximal voltage).

Release of ACh

Strips of plexus-containing longitudinal muscle (80–130 mg) were mounted in a 1 ml organ bath with platinum electrodes along the walls. The preparation was allowed to stabilize over a period of two hours of intermittent stimulation. During the release experiments the Tyrode contained eserine (3 μM) and was changed every 5 min. The preparation was electrically stimulated with biphasic pulses (0.5–3 Hz, 1 ms supramaximal voltage) for 280 s at 20 min intervals. Muscle contractions were recorded isotonically by means of a Grass FTO3 strain gauge transducer and a Grass polygraph. Collected bath fluid was immediately chilled, acidified to pH 6.5 and stored at -70°C until analyzed for ACh and choline (Ch) according to the method of Karlén *et al.* (1974). Briefly deuterated (D_6) ACh and Ch were added as internal standards, whereafter extraction was performed with dipicrylamine. After demethylation with sodium benzenethiolate the samples were extracted and run on LKB gas chromatograph-mass spectrometer ACh and Ch levels were calculated from peak height ratios between ions of deuterated and non-deuterated compounds. Accurate corrections were made for recovery from control samples containing known amounts of ACh and Ch. Apart from estimation by measurement of fragments m/e 58 and 64 the presence of fragments m/e 71 and 77 was confirmed as evidence for measurement of Ch derivatives.

Effector responses in whole segments of ileum

Segments of ileum (2–3 cm) with adjacent mesentery were mounted in a 50 ml bath and arranged for both transmural and periaxillary nerve stimulation. Contractions were elicited through electrodes parallel to and 5 mm apart from the preparation, and by Grass S88 stimulator delivering a single biphasic pulse (1 ms supramaximal voltage) each minute. The adrenergic nerves were electrically stimulated (3–10 Hz, 1 ms, 5–10 V) through periaxillary electrodes at appropriate intervals. Muscular activity was recorded isotonically as described above.

Drugs

Acetylcholine hydrochloride (Sigma Co., St. Louis, USA), adenosine (Sigma), angiotensin (Hypertensin Ciba-Geigy AG, Basle, Switzerland), atropine sulfate (Sigma), eserine sulfate (Sigma), guanethidine sulfate (Ciba-Geigy), histamine hydrochloride (ACO Stockholm, Sweden), 5-hydroxytryptamine creatinine sulfate (serotonin, Sigma), naloxone hydrochloride (End Labs, Garden City NY, USA), phentolamine hydrochloride (Ciba-Geigy), tetrodotoxin (Sigma). D_6 -ACh and D_6 -Ch were synthesized in the Department of Toxicology, Karolinska Institutet. All chemicals used were of reagent grade. Concentrations refer to the base unless otherwise stated.

Statistics

Statistical significance was tested according to Student's *t*-test for paired and unpaired series.

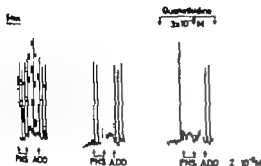


Fig. 6

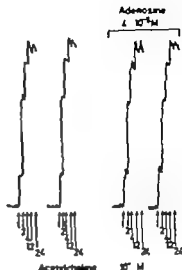


Fig. 7

Fig. 6. Isolated whole segment of the guinea pig ileum with adjacent mesentery. Contraction responses to transmural stimulation (single pulses, 1 sec, supramaximal voltage) at 1 min intervals. PNS = stimulation of mesenteric parasympathetic nerves (10 Hz, 1000 pulses, 1 sec, 5 V). ADO = adenosine 2×10^{-6} M added to bath fluid. Washing between repetitive periods. Constant magnification of responses throughout the exp. Guanethidine added to bath fluid as indicated by horizontal bar.

Fig. 7. Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses. Acetylcholine added to bath fluid in cumulative fashion giving total concentration as indicated at arrows. Washing between repetitive periods. Constant magnification of responses throughout the exp. Adenosine added to bath fluid as indicated by horizontal bar.

tion on contraction responses to transmural nerve stimulation. The inhibitory action of adenosine on responses to transmural stimulation was still present, however (Fig. 6).

Contraction responses of the longitudinal muscle were also obtained by increasing the potassium concentration of the bath fluid to 20 mM through addition of KCl. These responses were, when sensitive to atropine (3×10^{-6} M), inhibited by adenosine. Adenosine, at concentrations inhibiting responses to transmural nerve stimulation, did not alter contraction responses to exogenous ACh, which was administered in submaximal concentrations or cumulatively to give a complete dose-response curve (Fig. 7). After administration of tetrodotoxin ($3-6 \times 10^{-6}$ M), which blocked the contraction responses to transmural nerve stimulation, direct stimulation of the smooth muscle cells was obtained by prolonging the pulse duration to 20-30 ms. The ensuing contraction responses (uninfluenced by atropine $0.3-1 \times 10^{-6}$ M and guanethidine 3×10^{-6} M) were not altered by adenosine in concentrations causing at least 50% inhibition of neurogenic responses in the same preparation (4 expts.) (Fig. 8). Contraction responses to histamine were also uninfluenced by adenosine. On the other hand, serotonin (10^{-6} M) and angiotensin II (3×10^{-6} M) produced contraction responses, which were inhibited by adenosine in concentrations depressing responses to transmural nerve stimulation (Fig. 9).

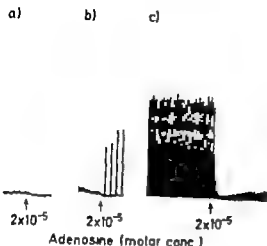


Fig. 4 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (1 ms, supramaximal voltage) at varied frequencies and intervals. a) 3 Hz, 15 pulses at 1 min intervals. b) Single pulses at 1 min intervals. c) Single pulses at 10 s intervals. Constant magnification of responses throughout the experiment. Wash at dots.

unaffected by naloxone, suggesting that it was not due to activation of opiate receptors (Fig. 1). Although there was some variation in reactivity between different preparations, a given dose usually caused very reproducible responses throughout an experiment. At a stimulation frequency of 3 Hz a significant inhibition of the contraction response was obtained with adenosine, $7 \cdot 10^{-6}$ M and the inhibition progressively increased with increasing doses of adenosine, approaching 80% at $2 \cdot 10^{-4}$ M (Fig. 2). Adenosine caused inhibition of both phasic ("twitch") and tonic contractions induced by transmural nerve stimulation (Fig. 3). Adenosine was more prone to inhibit the contraction response when the preparation was stimulated with single shocks each minute, and even more so when pulses were delivered continuously at 0.1 Hz (Fig. 4). At 1 pulse per minute $53 \pm 5\%$ (mean \pm S.E., $n = 9$) inhibition was obtained by adenosine $2 \cdot 10^{-4}$ M and at 0.1 Hz it was $84 \pm 4\%$ ($n = 8$). The difference between these two values is highly significant ($p < 0.001$). A common phenomenon was a tendency of adenosine-inhibited responses to slowly return towards the control level in spite the preparation had not been washed. In a few cases this was very prominent (Fig. 5), and repeated doses could be given, still eliciting comparable inhibition without interposed washing. This suggests elimination of the drug from its site of action or some other type of reactivation process.

Administration of guanethidine ($3 \cdot 10^{-4}$ M) or phentolamine ($3 \cdot 10^{-4}$ M) did not alter the inhibitory action of adenosine on contraction responses to transmural nerve stimulation. To ascertain a complete block of adrenergic neurotransmission, experiments were performed on whole segments of ileum prepared according to Finkleman (1930). Guanethidine ($3 \cdot 10^{-4}$ M) abolished or even reversed the inhibitory effect of perivascular nerve stimula-

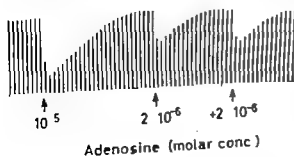


Fig. 5 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Preparation not washed during the experiment.

Table I Release of acetylcholine and choline from the everted longitudinal muscle of the guinea pig ileum, as determined by gas chromatography-mass spectrometry. Bath fluid as collected at 5 min intervals and transmural stimulation delivered for 280 at 0.5 or 3.11 every 20 min. Rest denotes the release during 5 min period just prior to stimulation. Values in pmol g^{-1} min $^{-1}$. Mean and S.E. of 6 experiments. Statistical significance tested according to Student's t-test for paired data (** $p < 0.01$)

	Control		Adenosine 2×10^{-4} M	
	ACh	Ch	ACh	Ch
1.5 Hz				
Rest	83 \pm 6	737 \pm 99	68 \pm 5	666 \pm 82
Stim	190 \pm 24	797 \pm 77	97 \pm 9**	681 \pm 76
3 Hz				
Rest	130 \pm 37	910 \pm 132	98 \pm 22	819 \pm 167
Stim	401 \pm 77	962 \pm 179	378 \pm 44**	899 \pm 167

at the 0.1% level according to Student's t-test. After washout of adenosine the overflow of ACh promptly returned to the control level (Table II).

The release of Ch was not significantly increased by transmural stimulation, and not altered by administration of adenosine. Ch release was higher (7.9 times) than ACh release during resting periods, and also during stimulation (1.5-7 times) (Table I).

Discussion

The present study has confirmed previous suggestions that adenosine inhibits ACh release from the guinea pig ileum (see Introduction). We have presented both indirect evidence and direct proof by chemical determination of ACh with gas chromatography-mass spectrometry. It was originally shown by Takagi and Takayanagi (1972) that different purine compounds, namely ATP, cyclic AMP and dibutyryl cyclic AMP inhibited contractions of the guinea pig ileum induced by electrical stimulation, serotonin and nicotine, while those induced by ACh were unaffected. Furthermore, ACh release (estimated by bioassay) following electrical stimulation was depressed during nucleotide administration. Later Glinzler and Vassicchio (1975) showed that the nucleoside adenosine was also able to inhibit electrically induced contractions of the guinea pig ileum, which has been concluded to be due to in-

Table II Net overflow of acetylcholine from the everted longitudinal muscle of the guinea pig ileum, evoked by consecutive periods of transmural nerve stimulation for 280 at 20 min intervals. Acetylcholine determined by gas chromatography-mass spectrometry. Net overflow was calculated by subtraction of the acetylcholine released during the 5 min period prior to stimulation from the amount released during transmural stimulation. Percent of control, number of experiments in brackets. Statistical significance tested according to Student's t-test for paired data (** $p < 0.001$)

	Series 1 (Control = 100 %)	Series 2 Adenosine 2×10^{-4} M	Series 3
0.5 Hz	100 (6)	27 \pm 7 (6)***	90 \pm 11 (6)
3 Hz	100 (6)	39 \pm 3 (6)***	106 \pm 8 (6)

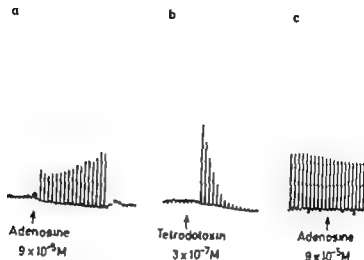


Fig. 8 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation before and during tetrodotoxin treatment. a) and b) Contraction responses to transmural stimulation with pulses of short duration (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. c) Direct smooth muscle activation by pulses of long duration (10 Hz, 50 pulses, 20 ms, supramaximal voltage) at 1 min intervals (magnification of contraction responses $\times 2$). Wash at dots.

Before eserization overflow of ACh in response to nerve stimulation could not be detected. In the presence of eserine ($3 \cdot 10^{-6}$ M), however nerve stimulation gave detectable overflow of ACh, which after 1 hour had a S_2 to S_1 ratio of 1.03 ± 0.11 ($n=8$) at 0.5 Hz and 1.00 ± 0.06 ($n=6$) at 3 Hz. The outflow of choline was little influenced by nerve stimulations and slowly decreased throughout the experiments. Adenosine ($2 \cdot 10^{-4}$ M) had no discernible effect on spontaneous outflow of ACh but significantly inhibited the overflow of ACh in response to transmural stimulation. The efflux of Ch was not significantly affected by this adenosine concentration (Table 1). Adenosine ($2 \cdot 10^{-4}$ M) inhibited the net overflow of ACh in response to nerve stimulation (overflow during nerve stimulation minus basal overflow) by 41% at 3 Hz and 73% at 0.5 Hz stimulation. This difference is significant.

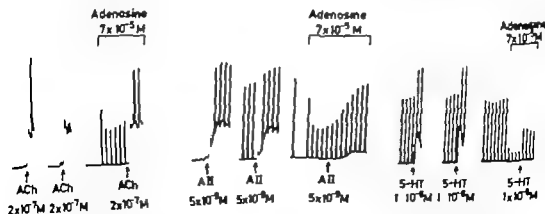


Fig. 9 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals and on contraction responses to added agonists. ACh—acetylcholine, A II—a glutathione II, 5-HT—serotonin. Wash at dots. Adenosine added to bath fluid as indicated by horizontal bars.

Table I. Release of acetylcholine and choline from the excised longitudinal muscle of the guinea pig ileum, as determined by gas chromatography-mass spectrometry. Bath fluid was collected (5 ml) between and (transmural) stimulation delivered for 280 s at 0.5 or 3 Hz every 20 min. Rest denotes the silent period (5 min) just prior to stimulation. Values in pmol g^{-1} min $^{-1}$ ACh and S.E. of 6 repes. Statistical significance tested according to Student's *t*-test for paired data ($^{**} p < 0.01$).

	Control		Adenosine 2×10^{-4} M	
	ACh	Ch	ACh	Ch
0.5 Hz				
Net	81 ± 4	757 ± 99	68 ± 5	666 ± 82
Spill	190 ± 24	797 ± 77	$97 \pm 9^{**}$	681 ± 76
3 Hz				
Net	138 ± 37	910 ± 132	98 ± 22	819 ± 167
Spill	301 ± 77	962 ± 179	$378 \pm 44^{**}$	899 ± 167

at the 0.1 level according to Student's *t*-test. After washout of adenosine the overflow of ACh promptly returned to the control level (Table II).

The release of Ch was not significantly increased by transmural stimulations, and not affected by administration of adenosine. Ch release was higher (7-9 times) than ACh release during resting periods, and also during stimulations (1.5-7 times) (Table I).

Discussion

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Table II. Net overflow of acetylcholine from the excised longitudinal muscle of the guinea pig ileum, evoked by consecutive periods of transmural nerve stimulation for 280 s at 20 min intervals. Acetylcholine determined by gas chromatography-mass spectrometry. Net overflow was calculated by subtraction of the acetylcholine released during the 5 min period prior to stimulation from the amount released during transmural stimulation. Percent of control, number of experiments in brackets. Statistical significance tested according to Student's *t*-test for paired data ($^{**} p < 0.001$).

	Series 1 (Control 100 %)	Series 2 Adenosine 2×10^{-4} M	Series 3
0.5 Hz	100 (4)	27 ± 7 (6) **	90 ± 13 (4)
3 Hz	100 (6)	39 ± 3 (6) **	106 ± 8 (4)

hibition of ACh release since responses to applied ACh were unaltered (Sawynok and Jhamandas 1976).

The inhibition by adenosine of contractile responses to nerve stimulation is not likely due to interactions with adrenergic mechanisms. The effect of adenosine is unaffected by phenolamine (Vizi and Knoll 1976 and present results) or by phenoxybenzamine (Sawynok and Jhamandas 1976). Furthermore our results demonstrate that guanethidine in a dose that completely eliminates the inhibitory effect of periaxillary nerve stimulation (presumably adrenergic) does not affect the response to adenosine. Finally adenosine is known to inhibit the release of noradrenaline in a number of tissues (Hedqvist and Fredholm 1976, Verbaegh *et al* 1977). Theoretically inhibition of noradrenaline release in the guinea pig ileum would tend to, if anything, increase ACh release and contractile responses would be enhanced (Paton and Vizi 1969).

It is well known that morphine gives a similar type of inhibition of contractile response as adenosine in the guinea pig ileum, presumably due to inhibition of ACh release (Paton 1957, Schaumann 1957). Indeed it has been suggested, referring to the actions of adenosine and morphine that "the biochemical pathways responsible for mediating the effects may share some common elements" (Gintzler and Musacchio 1975). Endogenous opioid peptide have profound actions in the guinea pig ileum (Waterfield *et al* 1977) and have been demonstrated to be released from the tissue by electrical stimulation (Schulz *et al* 1977). Therefore it is possible that the effect of adenosine could be mediated directly over morphine receptors or via the release of endogenous substances with morphine-like activity. However neither we nor Sawynok and Jhamandas (1976) could inhibit the action of adenosine by the morphine receptor antagonist naloxone, rendering this possibility less likely. On the other hand, the possibility that morphine might release an adenosine-like substance (Sawynok and Jhamandas 1976) must remain open.

In the rabbit small intestine adenosine, and related compounds, decrease tone and contractility by a mechanism apparently unrelated to nervous elements (Drury and Szek-Györgyi 1929, Euler and Gaddum 1931, McKenzie *et al* 1977, Ally and Nakatsu 1977). However in the guinea pig ileum the effect is in all probability due to an action on nerves. Thus the effect of exogenous ACh is unaffected by adenosine (Sawynok and Jhamandas 1976, present results), as are the effects of the directly acting agonist histamine (present results). Concentrations of adenosine that are highly effective on transmural stimulation with pulses of short duration are without effect on responses to stimulation with pulses of long duration after tetrodotoxin (Fig. 8), which directly activates the smooth muscle elements. Furthermore, the effects of several agonists supposed to act via release of ACh, *i.e.* potassium, serotonin (Rocha e Silva *et al* 1953, Gaddum *et al* 1957), angiotensin II (Robertson and Rubin 1962) and cholecystokinin (Vizi *et al* 1973), are inhibited by adenosine (Vizi and Knoll 1976, present results).

The best evidence for a presynaptic action of adenosine is the direct demonstration that it inhibited ACh release. Using bioassay (guinea pig ileum) Vizi and Knoll (1976) reported that $4 \cdot 10^{-6}$ M adenosine inhibited ACh release induced by 0.2 Hz. In a recent abstract Leighton and Parmeter (1977) state that adenosine inhibits 3 H ACh release from 3 H-ACh labelled guinea pig ileum. In the present study we report that the release of chemically

identified endogenous ACh is inhibited by adenosine. The inhibition of ACh release by adenosine was frequency dependent. 2×10^{-4} M adenosine inhibited ACh release by about 75% at 6.5 Hz but only by 40% at 3 Hz. Although results obtained with bioassay and electrical determination cannot be directly compared (Gustafsson *et al.*, to be publ.) the results of Vizi and Knoll (1976) suggest an even larger effect of adenosine at 0.2 Hz. In this context it should be mentioned that the inhibitory effect of adenosine on contractions due to electrical stimulation was dependent on the mode of stimulation used. Thus contractions due to single pulses were inhibited more than those due to short bursts at 3 Hz for 5 s. Inhibition was further increased if the interval between single pulses was reduced from 1 min to 10 s. It has been suggested that adenosine influences transmembrane calcium fluxes (Gardner *et al.* 1977) and it has also been proposed that presynaptic regulation of neurotransmitter release is ultimately dependent on altered calcium transport (*cf.* Westfall 1977) which normally triggers transmitter release (*cf.* Hubbard 1970). However the biochemical mechanisms underlying the frequency dependent inhibition of ACh release by adenosine and the dependence on the type of stimulation given are so far obscure.

Adenosine derivatives are released upon nerve stimulation in various parts of the gut (Burnstock *et al.* 1970, Satchell and Burnstock 1971, So *et al.* 1971). Adenosine is a major component hereof (Satchell and Burnstock 1971). Therefore it is possible that the described effect of adenosine on ACh release may be physiologically relevant. This possibility is independent on whether proposed purinergic nerves (Burnstock 1972) exist or not since it does not require that the purine compound subserves a transmitter function. Adenosine (or a related compound) could modulate ACh release irrespective of whether it is of nervous or non-nervous origin.

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Studies on the melanin-affinity of metal ions

By

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Abstract

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Melanin has a capacity to accommodate metal ions *in vivo* and *in vitro*. In the present study the relative melanin-affinity of various metal ions has been determined *in vitro* by use of the bisquaternary ammonium compound paraquat as a reference ion. Paraquat has previously been shown to bind to melanin with ionic bonding as the dominant mechanism of interaction. The relative melanin-affinity of the metal ions was determined by studying the ability of the metal ions to compete with paraquat for combining with the melanin. Pigment from beef eyes and synthetic melanin prepared from L-DOPA with tyrosinase were used. The affinity for melanin of the metal ions increased with increasing valencies. The order of affinity of the alkali metals was $\text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+$ and of the alkaline earth metals $\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$. Pb^{2+} had slightly higher affinity than the alkali metals. Pb^{2+} had the strongest affinity of the divalent cations and Cu^{2+} was next in order. Ni^{2+} , Co^{2+} and Mn^{2+} showed affinities in the mentioned order. La^{3+} and Ce^{3+} had a chemical affinity for the melanin, suggesting that it is the chemical and not the magnetic properties which determine the affinity. The studied metal ions showed the same order of affinity for the synthetic melanin as for the eye-melanin, suggesting that the protein moiety of the pigment granules plays a minor role in the binding of the metal ions. Melanin has been shown to contain numerous free carboxyl groups. The results are consistent with the hypothesis that the metal-ion binding of the melanin can be ascribed to cation-exchange activity with the free carboxyl groups in the melanin polymer as the main binding-sites.

Key words: melanin, cation exchanger, metal ions, paraquat.

Melanin has a capacity to bind metal ions *in vivo* and *in vitro* (Nicolais 1962, Cotzias and Papavasiliou 1964, Kikkawa *et al.* 1955, Selji *et al.* 1963, Stein 1955, Garner 1959, Taylor *et al.* 1964, Potts and Au 1971, Bruenger *et al.* 1967, Potts and Au 1976). This binding has been ascribed to a cation-exchange activity of the melanin (Bruenger *et al.* 1967, Potts and Au 1976), which in turn may be related to the presence of free carboxyl groups in the melanin polymer (Nicolais 1968). However, data on the relative affinity of various metal ions for melanin are still scarce and it has not been established whether there is a parallelism between the metal-binding capacity of melanin and of other carboxyl ion exchange resins. We have recently shown that the bisquaternary ammonium compound paraquat is bound on melanin *in vivo* and *in vitro* with ionic bonding as the dominant mechanism of the interaction (Larsson *et al.* 1977). Spectrophotometric determinations of the melanin-binding of paraquat

present in incubation media together with metal ions also gave information on the relative affinity of the metal ions for melanin. It became apparent that paraquat may be used as a reference ion to determine the relative affinity of metal ions for melanin. Methods based on the same principle, *i.e.* competition between organic and inorganic cations for the binding to a polyanion, have previously been used to study the relative affinity of cations to poly anions (Scott 1973 Simkiss and Tyler 1958). In the present investigation, the relative affinity of metal ions for pigment from beef eyes and for synthetic melanin prepared from L DOPA has been determined by use of paraquat as a reference ion.

Material and methods

Chemicals. Crystalline paraquat dichloride (N,N'-dimethyl-4,4'-dipyridylum dichloride) was a gift from the Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Cheshire, England. Other chemicals used in the study were of analytical grade and were purchased from regular commercial sources.

Pigment preparation. Pigment from beef eyes was prepared as described by Potts (1964). The final pigment granule suspension was adjusted to contain 10 mg by dry weight of pigment granules per ml suspension. It was stored at $+2^{\circ}\text{C}$ until used.

Synthetic melanin was prepared by dissolving 10 g L DOPA and 20 mg tyrosinase (polyphenol oxidase from mushrooms, 700 units/mg, Sigma Chemical Co., St. Louis, Missouri, USA) in 500 ml 1/15 M Sorensen buffer pH 6.8. After shaking in a water bath at 37°C for 17 h, during which period the solution was bubbled with air, the resulting black precipitate was collected by centrifugation and washed 4 times in distilled water. The yield was 29%. The final melanin suspension was adjusted to contain 5 mg by dry weight of melanin per ml suspension. It was stored at $+2^{\circ}\text{C}$ until used.

Experiments. The ability of metal ions to compete with paraquat for combination with melanin was studied by adding various amounts of the chloride salts of the metal ions to the incubation solutions containing melanin and paraquat. The amount of paraquat which had been bound on the melanin after 30 minutes incubation was then determined. The incubation procedure described by Potts (1964) was used with modifications (Larsson *et al.* 1977). The melanin suspension medium contained 10 mg pigment granules or 5 mg synthetic melanin, $3.57 \cdot 10^{-4}$ M paraquat ($= 3.57 \cdot 10^{-4}$ M paraquat) and the desired amount of metal chloride salt in 7 ml of distilled water. After the incubations, the pigment was sedimented by centrifugation at $35\,000 \times g$ for 10 min in an MSE 25 31 g/h Speed centrifuge and the paraquat remaining in the supernatant was measured spectrophotometrically in a Hitachi Perkin-Elmer 124 Spectrophotometer at 257 nm. All determinations were made in duplicate. The difference between the two determinations was found to be less than $\pm 0.2\%$. Since variations in pH may influence the binding of paraquat on melanin (Larsson *et al.* 1977), the salts were also added to distilled water at the same concentrations as used in the incubation experiments, and the pH values of these solutions were recorded by means of a PHM 62 Standard pH-meter with a glass electrode (Radiometer Copenhagen, Denmark).

Results

Experiments with eye-melanin. In initial experiments, the effect of various concentrations of Pb^{2+} , Ca^{2+} and Mg^{2+} on the binding of paraquat on melanin was determined. It was found that the binding of paraquat on melanin was strongly influenced by the ionic strength of the incubation media (Fig. 1). Pb^{2+} was more effective than Ca^{2+} which in turn was more effective than Mg^{2+} at all the concentrations studied. On basis of these experiments it was decided to study the inhibiting effect on the melanin binding of paraquat by the various metal ions in concentrations of $3.57 \cdot 10^{-4}$ M and 10^{-6} M. It was found that in general the extent of inhibition of the paraquat binding increased with increasing valencies of the metal ions (Table I). This was seen both at the high and low concentrations of the metal ions. The

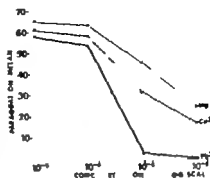


Fig. 1. The ability of Mg^{++} , Ca^{++} and Pb^{++} to inhibit the binding of paraquat on eye-oncogenes. 2.5 μ mole of paraquat ($3.57 \cdot 10^{-4}$ M) was incubated in 7 ml pigment granule (10 mg) suspensions containing various amounts of the chloride salts of Mg^{++} , Ca^{++} and Pb^{++} . After 45 min incubation, the binding of the paraquat on the pigment granules was determined.

order of inhibition among the various metal ions was also the same at both concentrations. Among the monovalent metal ions, Tl was slightly more effective than the alkali metals. The order of effectiveness of the alkali metals was $Cs > Rb > K > Na > Li$. Of the divalent metal ions, Pb^{++} and Cu^{++} in the mentioned order were more effective in inhibiting the paraquat binding than the other metal ions in this group. The order of effectiveness of

Table 1. The ability of metal ions to inhibit the binding of paraquat on eye-oncogenes.

	paraquat on suspension in 10^{-3} M metal ion solution	pH of 10^{-3} M metal ion solution	% paraquat on suspension in $3.57 \cdot 10^{-4}$ M metal ion solution	pH of $3.57 \cdot 10^{-4}$ M metal ion solution
control	64.3	6.0 ⁰⁰	64.3	6.0 ⁰⁰
Li	63.3	5.5	67.2	5.6
Na	59.7	5.7	66.8	5.7
K	58.3	5.3	66.5	5.6
Rb	57.2	6.3	65.9	5.9
Cs	52.6	5.7	63.8	6.2
Li	—	—	59.8	5.9
Na	24.8	5.4	54.7	5.4
K	17.0	6.0	45.6	5.7
Rb	14.2	5.4	45.3	5.3
Cs	12.5	5.4	41.3	5.4
Li	17.7	5.4	43.6	5.5
Na	14.1	5.6	39.6	5.4
K	11.2	5.4	37.0	5.5
Rb	4.2	5.0	32.0	5.4
Cs	0.6	5.2	26.3	5.3
Li	3.9	5.4	20.3	5.4
Na	3.0	6.5	18.7	5.5

control: no metal ion added
pH of distilled water
not soluble in 10^{-3} M concentration
2.5 μ mole of paraquat ($3.57 \cdot 10^{-4}$ M) was incubated in 7 ml pigment granule (10 mg) suspensions containing 10^{-3} M or $3.57 \cdot 10^{-4}$ M metal-chloride salts. After 45 minutes incubation, the binding of the paraquat on the pigment granules was determined.

TABLE II The ability of metal ions to inhibit the binding of paraquat on synthetic melanin.

Metal ion	% paraquat on melanin in $3.57 \cdot 10^{-4}$ M metal ion solution
—	98.7
Na	98.0
Ca	85.3
Ni^{2+}	72.9
Pb^{2+}	44.1
La^{3+}	23.3

control: no metal ion added

2.5 μmol of paraquat ($\approx 3.57 \cdot 10^{-4}$ M) was incubated in 7 ml of suspensions containing 5 mg synthetic melanin and $3.57 \cdot 10^{-4}$ M of metal-chloride salts. After 45 minutes incubation, the binding of the paraquat on the melanin was determined

the alkaline earth metals was $\text{Ba}^{2+} > \text{Sr} > \text{Ca}^{2+} > \text{Mg}^{2+}$. The order of the other divalent metal ions was $\text{Ni} > \text{Co} > \text{Mn}$. La and Gd showed a similar ability to inhibit the melanin binding of the paraquat.

The pH values of the solutions of the studied metal ions showed only small variations (Table I). It was obvious that these variations had no relation to the inhibiting capacity of the metal ions. Originally Cr^{3+} , Al and Sn^{4+} were included in the study. It was found, however, that these cations by hydrolysis lowered the pH. They will therefore be present in the water as complex ions with water molecules and OH^- . The ability to inhibit the paraquat binding will then be caused by the complex ions and the H^+ ions released by the hydrolysis and not by the pure metal ions. These metal ions were therefore excluded from the study.

Experiments with synthetic melanin. Paraquat was more effectively bound on the synthetic melanin than on the eye melanin (Table II). As with the eye melanin, the metal ion induced inhibition of the paraquat binding increased with increasing valencies of the metal ions. The order of effectiveness of the studied metal ions was the same as that observed with the eye-melanin.

Discussion

The results of the present study have shown that the attraction to the melanin of the metal ions increases with increasing valencies. This is a general characteristic of cation exchange reactions and is due to the electrostatic fields of the cations (Scott 1968). The order of affinity for the alkali metals was found to be $\text{Cs} > \text{Rb} > \text{K} > \text{Na} > \text{Li}$ and for the alkaline earth metals $\text{Ba} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}$. The adsorbability of these ions is related to their hydrated ionic radius and follows the Hofmeister or lyotropic series, which is a general order obeyed for many ion exchange systems (Kunin 1958). Bungenberg de Jong (1949) studied the affinity of metal ions for carboxyl colloids (Na-arabinate and Na-pectinate), using the so called reversal-of-charge technique and found the order of affinity of the alkali metals to be $\text{K} > \text{Na} > \text{Li}^+$ and for the alkaline earth metals $\text{Ba} > \text{Sr}^{2+} > \text{Ca} > \text{Mg}$. Haug (1961) who worked with alginates (which carry carboxyl groups) using H^+ as reference ion, found the same order of affinity for the alkaline earth metals. Thus, these findings correspond

to observations made in the present study. In their studies, Bungenberg de Jong (1949) and Haug (1961) found that of the divalent cations Pb^{2+} had the strongest affinity and that Cu^{2+} was next in order. These findings also correspond to the results of the present study. Bruenger *et al.* (1967) have reported a strong affinity for melanin of ^{210}Pb . A strong affinity of Cu for melanin was observed by Potts and Au (1976). Synthetic carboxyl resins have been reported to have a high affinity for Cu^{2+} (Kunin 1958). It has been shown that melanin contains a stable free radical (Commoner *et al.* 1954). Blois *et al.* (1964) showed that Cu^{2+} which is paramagnetic, eliminated the free radical signal. In the present study the melanin affinity of La^{3+} and Gd^{3+} was studied. La^{3+} is diamagnetic and Gd^{3+} is paramagnetic. Serna *et al.* (1976) showed that the free radical signal of melanin is blocked by Gd^{3+} but not by La^{3+} . Since La^{3+} and Gd^{3+} have similar chemical properties and Gd^{3+} and Cu^{2+} have similar magnetic properties, it was concluded that magnetic and not chemical interaction explains the loss of free radical signal (Serna *et al.* 1976). Studies by Baldry and Swan (1977) indicated that the free radicals of melanin were inaccessible to chemical attacks by reagents such as metal ions. In the present study La^{3+} and Gd^{3+} showed similar affinity for melanin. This indicates that it is the chemical and not the magnetic properties of the metal ions which determine the extent of the binding to the melanin. This result supports the conclusions of Serna *et al.* (1976) and Baldry and Swan (1977).

The experiments with synthetic melanin showed that the studied metal ions had the same order of affinity as for eye-melanin. This suggests that the protein moiety of the melanin plays a minor role in the binding of the metal ions.

The results of the present study support the hypothesis that the metal ion binding of melanin is determined mainly by the free carboxyl groups present in the melanin polymer and melanin will in this respect behave like a weak acid cation-exchanger. However a high affinity of a metal ion for melanin *in vitro* does not necessarily mean that a high uptake occurs also *in vivo*. Melanin is an intracellular constituent, and transport barriers at the cell membranes may prevent metal ions from reaching the melanin *in vivo*. Inversely an effective transport mechanism may promote the uptake in the melanin containing tissues.

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Sympathetic innervation of the urinary bladder and urethral muscle in the pig

By

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Abstract

LARSEN, J. J., J. NORDLING and B. CHRISTENSEN. Sympathetic innervation of the urinary bladder and urethral muscle in the pig. *Acta physiol. scand.* 1978. 104. 485-490.

The sympathetic innervation of the lower urinary tract was studied in the female and male pig using specific histochemical techniques for visualization of noradrenaline containing nerves. In smooth muscle tissue the detector few evenly distributed adrenergic nerve terminals were found. A greater number of terminals were observed in the trigone, bladder neck and urethra. There was no sex difference. In smooth muscle specimens from pig detrusor and trigone sympathetic nerve terminals were more abundant than in corresponding tissue from boar.

Key-words: Pig, urinary bladder, sympathetic innervation, fluorescence histochemistry.

The autonomic control of the function of the lower urinary tract is very complex. There are at least two efferent reflexes which are important for normal continence and micturition mechanisms (Mahony *et al.* 1977). Sympathetic as well as parasympathetic nerves are involved in these reflexes. Adrenergic and cholinergic nerve terminals have been demonstrated in the smooth muscle of the bladder and urethra in different species, including man, by means of specific histochemical techniques (El-Badrawi and Schenk 1966, Nordén *et al.* 1976, Ek *et al.* 1977 a, Sundin *et al.* 1977). Studies of adrenoreceptors carried out *in vitro* and *in vivo* in several animal species and in man have revealed the distribution of α - and β -adrenoreceptors in these organs (Edvardsen and Serckleiv 1968, Awad *et al.* 1974, Ek *et al.* 1977 b, Hindmarsh *et al.* 1977). Significant species variations have been shown indicating differences in the functional importance of the sympathetic nervous system in the lower urinary tract from animal to animal and from animal to man (El-Badrawi and Schenk 1966, Edvardsen and Serckleiv 1968, Nergårdh *et al.* 1977). Recently the pig has, with advantage, been taken into use for experimental purposes in studies of the kidney, ureter and bladder (Gyrd-Hansen *et al.* 1969, Djurhuus *et al.* 1976, Larsen and Mortensen 1978). The structure of the detrusor muscle of the pig has been described by Larsen (1977), and the

TABLE 1 Evaluation of the sympathetic innervation of the smooth muscle in the pig lower urinary tract ($n=10$) based on the distribution of adrenergic nerve terminals according to the following score system

- 0 No adrenergic nerve terminals observed in the muscle.
- 1 Less than one adrenergic nerve terminal present per smooth muscle bundle.
- 2 On the average one adrenergic nerve terminal in each smooth muscle bundle.
- 3 On the average 2-5 adrenergic nerve terminals in each smooth muscle bundle.
- 4 On the average one adrenergic nerve terminal at each smooth muscle cell (i.e. 5-10 nerve terminals per muscle bundle) corresponding to the picture seen in control sections obtained from the ductus deferens of the rat.

Pig no.	1	2	3	4	5	6	7	8	9	10	\bar{x}
Sex	♀	♀	♀	♂	♂	♀	♂	♀	♀	♀	
Detrusor anterior	1	1	1	1	1	1	1	2	2	2	1.3
Detrusor right side	0	1	1	1	1	1	1	2	1	1	1.0
Detrusor left side	1	1	1	1	1	2	2	1	2	1	1.3
Detrusor posterior	1		1	0	1	1	2	1	1	1	1.0
Trigone area	3	3		2	2	3	3	2	3	3	2.6
Bladder neck	1	3	4	1	4)	3	3	3	3	2.8
Urethra)	3)	3	3	3	2	3	3	2.8

) Specimen with too small amount of smooth muscle for evaluation.

presence and type of β -adrenoceptors in the detrusor muscle of pig and man have been investigated (Larsen 1978). The present study was carried out in order to evaluate quantitatively the morphology of the sympathetic innervation of the lower urinary tract of the pig and to compare the results with previously reported findings in man (Nordling and Christensen 1978).

Methods

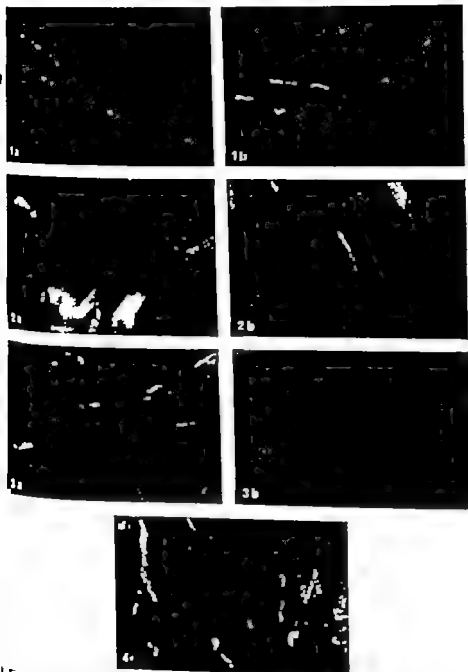
Specimens from seven female and three male (boars) pigs weighing about 90 kg were obtained at a slaughter house. The bladder and urethra were removed *in toto*, in the cases 25 ml and in one case (pig no. 1) immediately after death. Specimens of smooth muscle were removed from the anterior, posterior and lateral walls of the detrusor from the trigone, bladder neck, and urethra just above the pelvic floor. The muscle pieces were frozen in liquid propane cooled by liquid nitrogen according to the method described by Falck *et al.* (1962). The specimens were freeze-dried for 5-7 days, then exposed to formaldehyde vapour at 20°C for 1 h, imbedded in paraffin and cut in 10 μ m thick sections. During this treatment catecholamines are converted to strongly green fluorescent products (Falck *et al.* 1962). Sections were investigated by fluorescence microscopy before and after addition of freshly prepared 0.03 sodium borohydride (NaBH_4) in 95% isopropyl alcohol. Addition of NaBH_4 causes the specific catecholamine fluorescence to disappear within a few seconds—a process which can be observed in the microscope. By this method the specific catecholamine fluorescence can be distinguished from the persistent non-specific fluorescence (e.g. autofluorescence) of elastic fibres etc. (Corrodi *et al.* 1964; Nordling and Christensen 1978).

A Leitz orthoplan fluorescence microscope supplied with an ultra high pressure mercury lamp (Osram HBO 200) and equipped with a Leitz excitation filter BO 3 + S 405 and an emission filter K 490 was used. Photomicrographs were taken on Kodak Pan Estar AH 35 mm roll films with an exposure time from 20 to 30 s. The processing of the films was modified to give film sensitivity equivalent to 800 ASA.

A score system was used to quantitate the distribution of adrenergic innervation (see legend to Table 1).

Results

Table 1 shows the results obtained according to the score system. Adrenergic nerve terminals were observed in the smooth muscle of all parts of the lower urinary tract examined. There



- Fig. 1 Smooth muscle from the urinary bladder neck of female pig (a) and male pig (b). Some adrenergic nerve terminals are present. Fluorescence microphotography $\times 300$.
- Fig. 2 Smooth muscle from the anterior part of the detrusor of pig no. 1 (a) and pig no. 2 (b). An adrenergic nerve terminal is present in each picture. Fluorescence microphotography $\times 300$.
- Fig. 3 Smooth muscle from the trigone area before (a) and after (b) the addition of sodium borohydride. The disappearance of adrenergic nerve terminals is illustrated. Fluorescence microphotography $\times 300$.
- Fig. 4 Smooth muscle from the proximal part of the urethra. Some adrenergic nerve terminals are present. Fluorescence microphotography $\times 300$.

TABLE I Evaluation of the sympathetic innervation of the smooth muscle in the pig lower urinary tract ($n = 10$) based on the distribution of adrenergic nerve terminals according to the following score system

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) Specimen with too small amount of smooth muscle for evaluation

presence and type of β -adrenoceptors in the detrusor muscle of pig and man have been investigated (Larsen 1978). The present study was carried out in order to evaluate quantitatively the morphology of the sympathetic innervation of the lower urinary tract of the pig and to compare the results with previously reported findings in man (Nordling and Christensen 1978).

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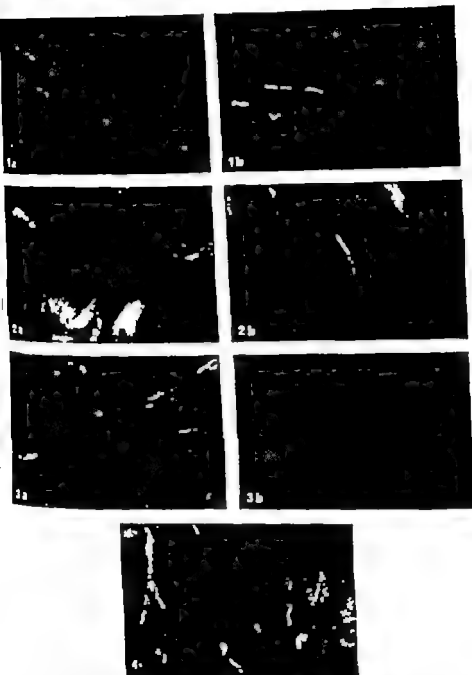


Fig. 1 Smooth muscle from the urinary bladder neck of female pig (a) and male pig (b). Some adrenergic nerve terminals are present. Fluorescence microphotography $\times 300$.

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Fig. 3 Smooth muscle from the trigone area before (a) and after (b) the addition of sodium borohydride. The disappearance of adrenergic nerve terminals is illustrated. Fluorescence microphotography $\times 300$.

Fig. 4 Smooth muscle from the proximal part of the urethra. Some adrenergic nerve terminals are present. Fluorescence microphotography $\times 300$.

was no difference between female and male pigs in any of the regions investigated (Table I and Fig. 1 a and 1 b).

No visible difference in number of nerves or intensity of fluorescence was seen between specimens obtained immediately after the death of the animal and specimens obtained 25 min after killing (Fig. 2 a and 2 b).

In spite of some variation the sympathetic nerves were clearly more abundant in the smooth muscle of the trigone (Fig. 3 a), bladder neck (Fig. 1 a and 1 b) and urethra (Fig. 4) than in the detrusor muscle (Fig. 2 a and 2 b). The number of adrenergic nerve terminals was almost equal in the trigone, bladder neck and urethra. The distribution of the sympathetic innervation of the detrusor muscle was without regional variations.

Generally the adrenergic nerve terminals in the smooth muscle examined were running parallel to the muscle cells. Transverse or oblique orientation was seldomly observed. Sympathetic terminals were abundant in the smooth muscle of blood vessels, but this innervation was carefully excluded in the present evaluation. No ganglionic cells were seen in any of the preparations.

Discussion

Differentiation between catecholamine fluorescence and autofluorescence based on the morphology and colour of the fluorescent product was found to be uncertain. However by using NaBH (Fig. 3 a and 3 b) an evaluation of the distribution of the adrenergic innervation based on the observation of the specific catecholamine fluorescence was considered to be reliable.

In a previous work it has been shown that the smooth muscle of the human detrusor and trigone has a very scarce sympathetic innervation (score 0 with the present score scale) (Nordling and Christensen 1978). Others have reported the same finding in smooth muscle of human detrusor, trigone and urethra (Ek *et al* 1977 a, Sundin *et al* 1977, Gosling and Dixon 1977) although some reported more abundant adrenergic nerves in the trigone (Ek *et al* 1977 a, Sundin *et al* 1977) and preprostatic male urethra (Gosling and Dixon 1977).

Species differences in the sympathetic innervation of the lower urinary tract have been demonstrated by several workers. El-Badawi and Schenk (1966) showed a pronounced difference in the distribution pattern of the adrenergic nerve terminals in the smooth muscle of the bladder from cat and dog on the one hand and rat and guinea-pig on the other hand. The number and general distribution of adrenergic nerves in the human bladder reported by Ek *et al* (1977 a) and by Sundin *et al* (1977) has by the former workers been found in accordance with the findings in animal species such as cat, dog, rabbit, rat and guinea-pig (Hamberger and Norberg 1965, El-Badawi and Schenk 1966, Owman and Sjöberg 1972, Wakade and Kirpekar 1972, Ratzer *et al* 1973, Sundin and Dahlström 1973, Dixon and Gosling 1974, Alm and Elmér 1975, Ratzer *et al* 1976). The scarce sympathetic innervation found in the human urethra (Ek *et al* 1977 a) differed from the rich one in e.g. cat, dog, guinea pig and rat (Owman *et al* 1971, Owman and Sjöberg 1972, Sundin and Dahlström 1973, Benson *et al* 1976). The very scarce sympathetic innervation of the smooth muscle of the human bladder neck and female proximal urethra found by Gosling *et al*

(1977) differed from the rich one found in the cat, dog, rabbit and rat (Hamberger and Norby 1965, El-Badawi and Schenk 1974, Gosling and Dixon 1975). It should however be mentioned that in most of the cited studies no scoring system for quantitation of the innervation has been used, which makes comparisons difficult.

The finding of a scarce but constant amount of adrenergic nerves in the detrusor muscle and a rich supply in the trigonal muscle of the pig differs from previous findings in human material using exactly the same technique (Nordling and Christensen 1978). In this study no score system was applied mainly because the adrenergic nerve supply of the smooth muscle is so scarce that all preparations would have obtained score 0 with the score scale used in the present study.

It can therefore be concluded that the adrenergic nerve supply of detrusor and trigonal smooth muscle is more abundant in the pig than in the human. Experimental results from the pig concerning physiology of the lower urinary tract, especially in respect to sympathetic innervation, can therefore only with great caution be extended to human physiology.

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Effect of synthetic substance P on internal carotid artery blood flow in man

By

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Abstract

Sännelund, H., L. Thulin, G. Tydén, C. Johansson, O. Muthreck and C. Börklund
Effect of synthetic substance P on internal carotid artery blood flow in man. *Acta physiol. scand.* 1978, 104: 491-495

Internal carotid artery blood flow was studied by peroperative electromagnetic blood flow measurement in 7 patients following 9 intravenous infusions of synthetic substance P $2 \text{ ng kg}^{-1} \text{ min}^{-1}$. The blood flow responses varied interindividually. No significant blood flow changes were observed for the whole group. The arterial mean blood pressure decreased significantly.

The circulatory effect of substance P (SP) has been extensively studied both in animals and in man since the initial report on SP was published by Euler and Gaddum (1931). SP has been found to influence the circulation with a transient fall in arterial blood pressure, both in animals and in man (Euler and Gaddum 1931, Pernow 1953 (a), Löfström *et al.* 1965, Maxwell 1968, Hallberg and Pernow 1975, Burcher *et al.* 1977, Eklund *et al.* 1977, Melchiorri *et al.* 1977). The fall in pressure is the result of a peripheral vasodilatation with increased blood flow in skin, muscle, adipose tissue (Pernow and Rosell 1975) and in the splanchnic vessels (Hallberg and Pernow 1975). Substance P appears to act directly on the vascular smooth muscle (Löfström *et al.* 1965, Eklund *et al.* 1977).

The occurrence of SP in the brain tissue (Pernow 1953 (b), Amin *et al.* 1954, Arnaudović *et al.* 1963, Laszlo 1963, Bakdau *et al.* 1968, Otsuka *et al.* 1975, Mroz *et al.* 1977, Hökfelt *et al.* 1977), and its potent vasodilating effect has focused our interest on the effect of i.v. administered synthetic SP on the internal carotid artery blood flow in man. A small pilot study indicated that internal carotid artery blood flow increased in normotonic patients following SP (Thulin *et al.* 1977). The present work reports an extended investiga-

Material

The investigation was performed in 7 patients (5 males and 2 females) with a mean age of 67 years (range 59–72 years), who were accepted for internal carotid artery (ICA) surgery. Indication for operation was based on clinical symptoms, mostly transient ischemic attacks, and on angiographical findings of obstructive disease of the carotid bifurcation. All patients suffered from arteriosclerosis. In 3 patients the ICA operated upon was stenosed by more than 50 per cent of the diameter of the lumen. Three patients suffered from hypertensive disease. No one had previously suffered from cardiac infarction or atrial fibrillation.

Methods

Internal carotid artery mean blood flow ml/min, was measured peroperatively with the aid of a square-wave electromagnetic flowmeter. An extravascular shunt (Elexström 1968) was used during the reconstruction. The blood measurements were performed after the arterial reconstruction during neurolept anaesthesia with N_2O , O_2 , fentanyl and droperidol.

The flowprobe was placed on the common carotid artery and the external carotid and the superior thyroid arteries were occluded during measurement. Zero flow line was obtained by occlusion of the vessel.

Brechial artery mean blood pressure mmHg, was measured by a mechano-electric pressure transducer. Both ICA flow and arterial blood pressure were continuously registered on a direct writing recorder.

Arterial carbon dioxide tension, ($PaCO_2$), kPa, was measured with the aid of a Severinghaus electrode. It was controlled every minute during measurements and kept constant by artificial ventilation.

Synthetic Substance P (Fisher *et al* 1974) dosage $ng\ kg^{-1}\ min^{-1}$ was given i.v. during ICA mean blood flow and arterial mean blood pressure were continuously measured during 5 min.

Statistical methods

Standard methods for statistical calculations of the data were employed (Snedecor and Cochran 1967). The following probability (p) levels of significance were used: $p < 0.001$, $p < 0.01$, $p < 0.05$.

Results

ICA blood flow, arterial mean pressure and $PaCO_2$ were studied in 7 patients after 9 i.v. infusions of SP. The results are presented in Fig. 1.

ICA mean blood flow was $188 \pm 8\ SD\ 59\ ml/min$ before administration of SP. The ICA blood flow increased after 4 infusions of $2\ ng\ kg^{-1}\ min^{-1}$ of SP and decreased or remained unchanged after 5 infusions. The mean ICA blood flow for the whole group was not significantly changed during the 5 min studied. There was no difference between hypertensive or normotensive patients.

Arterial mean blood pressure was $129 \pm 8\ SD\ 27\ mmHg$ before SP administration and decreased significantly within 1 min to $110 \pm 8\ SD\ 40\ mmHg$ ($p < 0.01$). The arterial mean pressure returned to control level within 2 min.

Arterial carbon dioxide tension was $4.0 \pm 0.5\ SD\ 0.6\ kPa$. It was not significantly changed during the procedure.

Discussion

As far as known, there are no reports in the literature concerning the influence of SP on cerebral blood flow (CBF) or ICA blood flow in man except for the previously mentioned pilot study (Thulin *et al* 1977).

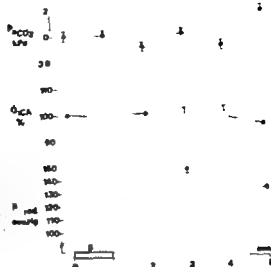


Fig. 1. Arterial CO_2 tension (P_{aCO_2}), kPa, ICA mean blood flow (\dot{Q}_{ICA}) as per cent of basal flow and radial artery mean blood pressure (P_{rad}), mmHg, before and after intravenous administration of $2 \text{ ng kg}^{-1} \text{ min}^{-1}$ of SP during 1 min. Vertical range bars indicate standard error of mean. Asterisks indicate probability levels of significance (p).

Electromagnetic flowmetry after arterial reconstruction of the carotid artery has been used to describe the interaction between central hemodynamics and cerebral circulation as reflected by ICA blood flow when arterial carbon dioxide tension, posture and physical activity are varied (Sampegård 1974). We have used this method perioperatively since the ICA flow changes most probably reflect changes in the cerebral blood flow of the tissue supplied by the artery (Sampegård and Carlens 1974). Furthermore, electromagnetic flowmetry allows registration of very rapid and brief flow changes.

Bullberg and Pernow (1975) studied the effect of SP on common carotid artery flow in the dog. They found about 50 per cent flow increase after continuous infusion of $2.4\text{--}3.6 \mu\text{g kg}^{-1} \text{ min}^{-1}$ of SP. This includes flow changes both in the internal and external carotid artery. In the dog only a minor part of the common carotid artery blood flow supplies the brain. Thus, the flow increase noticed by these authors may be the result of dilatation of the vessels to muscles and skin of the skull and no conclusions concerning the flow to the brain can be drawn.

The responses in blood pressure and ICA blood flow following SP varied considerably between the different patients. In 3 patients the arterial mean blood pressure was unchanged. In two of them the ICA blood flow remained unchanged and in one the blood flow decreased slightly. In two others ICA flow decreased by 15–20 per cent, presumably secondary to a pressure drop down to 55–70 mmHg, which values appear to be below the pressure level for cerebral circulatory autoregulation (Lassen 1959). In two patients ICA blood flow increased 15 per cent in spite of a decrease in arterial mean pressure to 90 mmHg from 110 and 125 mmHg respectively. The ICA blood flow increase in these patients indicates true vasodilatation in the cerebral vascular bed. But, on the other hand, the ICA flow decreased more than 10 per cent in two patients without significant pressure changes, suggesting a vasoconstriction.

The well documented vasodilating effect of SP on the peripheral vascular bed was also found in this study by a significant decrease in mean arterial blood pressure for the whole

group following infusion of SP. If SP has a direct effect on the vascular smooth muscle (Löfström *et al* 1965 Eklund *et al* 1977) a dilatation of the cerebral vessels with CBF increase would be expected. Minor increase in total CBF does not necessarily result in significant increase in ICA blood flow but a flow reduction as found in some patients, is definitely not expected.

Judging from the present study SP has a vasodilating effect in the peripheral vascular bed, but no similar effect on the cerebral vascular bed. The previous observations of a possible role for SP as a cerebral vasodilator must therefore be considered less significant.

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Acute effects of pressure on resistance vessel geometry

By

BJÖRN FOLKOW

The structurally set dimensions of inner radius (r_i) and wall thickness (w) are obviously of major importance for resistance vessel function, and even more so the ratio between them w/r_i , particularly concerning the altered hemodynamics in hypertension (e.g. Folkow 1954, 1978). It is known since over 100 years that hypertension is associated with hypertrophic thickening of the bulky arteriolar media, a microvascular analogue to left ventricular hypertrophy. However, the hemodynamic importance of this structural adaptation was for long overlooked, partly because it could first recently be precisely measured. The reason is that even modest shifts in smooth muscle activity and distending pressure, most difficult to avoid for microvessels, affect both w and r_i though in opposite directions, implying profound w/r_i changes and hence great errors.

It was first when techniques were introduced for standardization of the w/r_i balance that precise morphometric arteriolar comparisons became possible, thanks to Short (1966), Furuyama (1962) and Suwa and Takahashi (1971). When combined, their measurements in human primary hypertension show a structural r_i reduction associated with a media adaptation, so as to increase the w/r_i ratio largely in proportion to the pressure elevation. These changes were more marked in splanchnic and renal vessels than in muscle ones. Further close to the capillary level the increased w/r_i tapers off, suggesting that the smallest precapillary vessels are partly protected from the pressure elevation thanks to the increased 'upstream' resistance. Recently Mulvany, Aalkjaer and Hansen (1978) arrived at similar results on isolated mesenteric arterioles from spontaneously hypertensive rats (SHR) and normotensive controls (NCR). Measured at equal distending pressures and complete relaxation, the media thickness was increased about 50 per cent and r_i reduced 15 per cent in SHR, if anything elevating the structurally set w/r_i more than pressure was raised in this vascular bed like in man. — These morphometric findings fully confirm hemodynamic estimations of the altered resistance vessel design in hypertensive man and rats showing, among other things, a structurally determined average r_i reduction, despite exposure to a raised distending pressure (Folkow 1956, 1978).

However, recently Bohlen and Lobach (1978) performed *in vivo* morphometric estimations of r_i and w in a skeletal muscle (cremaster) vascular bed of SHR and normotensive controls (WKY). Though results varied between consecutive vascular sections, there was on an average no clear signs of any structural w/r_i increase even in established SHR hypertension. It is not unlikely however that the distal microvessels studied ($r_i \sim 50 \mu\text{m}$ or below)

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Peptide neurons in the vagus, splanchnic and sciatic nerves*

By

JAN AL LUNDBERG, TOMAS HÖRTELT, GÖRAN NILSSON, LARS TERENIUS,
JENS REYFELD, ROBERT ELDE, SAME SAID

Peripheral nerves in general contain both motor and sensory fibres. The classical transmitter substances in the motor nerves are either acetylcholine (Loewi, 1911) or noradrenaline (Euler 1956), although a non-cholinergic, non-adrenergic component also has been described (see Burnstock 1977). In addition peptides have been observed e.g. in extracts of the vagus nerve, including substance P (SP) (Euler 1963), vasoactive intestinal polypeptide (VIP) (Seld and Rosenberg 1976), gastrin (GAS) (Uvnäs-Wallensten *et al.* 1977) and somatostatin (SOM) (Uvnäs-Wallensten *et al.* 1978). In the present communication 3 different types of peripheral nerves were studied by the indirect immunofluorescence technique using antisera to several peptides. With this approach it was attempted to define further the cellular localization of such peptides.

10 male albino rats (b.wt. 150-200 g) and 10 male guinea pigs (b.wt. 300-400 g) were subjected to the following treatments according to the techniques described by Dahlström (1977). The vagus nerve was ligated at the cervical level, the greater splanchnic nerve at the subdiaphragmatic level and the sciatic nerve one cm below the foramen infrapiriforme. In 5 other animals of each kind colchicine (20%) or vinblastine (1%) were applied locally on the nodose ganglion and on the first sacral spinal ganglion. These drugs and the ligation technique were used to increase the levels of the peptides, since in untreated animals the cell bodies and axons often contain too low amounts to be visualized with immunocytochemistry. 24 h after both types of treatment the animals were perfused with icecold 4% paraformaldehyde and the nerves and ganglia were processed for the indirect immunofluorescence technique (see Codere 1958). Briefly cryostat sections were incubated with an antiserum raised to one of the peptides, SP, SOM, VIP, GAS and methionine-enkephalin (met-ENK) followed by a second incubation with fluorescein-isothiocyanate conjugated antibodies and examined in a Zeiss fluorescence microscope. The different antisera pretreated with an excess of the respective peptide served as control sera. For further details on experimental procedures including characteristics of antisera, see Lundberg *et al.* (1979). It should, however, be pointed out that the GAS antiserum also cross-reacts with cholecystokinin (CCK).

*Part of these results are communicated at the 6th EFP workshop in Znoj, Switzerland, January 1978.

and 60 per cent higher at 150 mmHg due to lower vascular distensibility in SHR (B vs D), this w/r_1 difference is marginal only (8 per cent) when normotensive vessels at 100 mmHg are compared with hypertensive ones at 150 mmHg (A vs D). The reason is the great reduction of w/r_1 when distension increases r_1 but decreases w which also the A/B and C/D relationships illustrate. Resistance is, however still 35 per cent higher in D vs A.

It might be argued that morphometric comparisons of hypertensive and normotensive vessels should better be performed at their ordinary pressures. However this would easily be misleading, as shown by the over 30 per cent w/r_1 reduction and over 20 per cent r_1 increase when normotensive vessels are, without other changes, exposed to the 150 mmHg acting on SHR vessels (A vs B). To acutely compensate for this passive 55 per cent reduction of resistance, a considerable smooth muscle activation would be needed in NCR, even to keep resistance the same as at 100 mmHg. The crucial difference to SHR is that the SHR resistance vessels can at 150 mmHg maintain a 35 per cent higher resistance than NCR at 100 mmHg without any accentuation of smooth muscle activity (Fig. 1). The more prominent the structural w/r_1 elevation, the more marked this ability of hypertensive vessels to maintain a high resistance at low smooth muscle activity where, as mentioned, splanchnic vessels in both man and SHR seem to be even more 'efficient' than muscle ones.

In conclusion the *in vivo* results of Bohlen and Lobach, as compared with those derived from earlier morphometric measurements *in vitro* and from quantitative hemodynamic analyses, may only be apparently in conflict and may in fact, coincide fairly closely concerning w/r_1 values, if only the most important influence of wall distensibility is taken into account. It follows that morphometric measurements must be performed at strictly equal distending pressures to allow for direct comparisons of w/r_1 values and, further deal with precisely corresponding precapillary sections, since the structural w/r_1 increase tends to taper off close to the capillary level. The results also emphasize the crucial hemodynamic importance of resistance vessel design with respect to its acute and chronic changes upon pressure alterations, and its great relevance for wall tension per unit wall layer.

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met-ENK-like peptide may be contained in axons of motor fibres. The nature of the fibres containing the peptides in the splanchnic nerves is at present under investigation. Similar distribution patterns have also been observed in immunohistochemical studies on these three types of nerves in the cat (Landberg, Uvnäs-Wallensten, Hökfelt *et al.* in preparation). Thus, in addition to the previously identified SP and SOM immunoreactive cells in sensory ganglia (Hökfelt *et al.* 1976) two further subpopulations have been identified containing a GAS-(CCK)-like and a VIP-like peptide, respectively. Finally ENK-like material is present in peripheral nerves with a probable central origin. The localization of the peripheral innervation areas and of putative opiate receptors of such ENK-positive fibres projecting e.g. in the sciatic nerve remains to be elucidated.

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Fig. 1 A-C. Immunofluorescence micrographs of sections of the vagus nerve (A) and sciatic nerve (B) and the nodose ganglion (C) of the rat 24 h after ligation (A, B) or colchicine application (C) incubated with antiserum to SP (A, C) and met ENK (B). Many SP (A) and some met ENK (B) positive fibres are seen above the ligation and some met ENK positive ones below (B). Small and large (arrows) SP positive cells are seen in the nodose ganglion. Arrow heads in A and B indicate site of ligation. Bars indicate 50 μ m.

SP, SOM, VIP, GAS- and met ENK immunoreactive material were found in all three nerves studied as shown by their accumulation central (and in the sciatic nerve, partly peripheral) to the ligations, indicating a rapid axonal transport (Fig. 1 A, B). In Table I, subjective estimations have been made to schematically indicate the number of peptide containing nerves above a ligation in the guinea pig. In the rat similar distribution patterns were observed although the number of nerves appeared to be slightly lower. In the nodose ganglion high numbers of small and some large SP immunoreactive cell bodies were observed (Fig. 1 C). Moderate numbers of VIP immunoreactive cells but only few SOM and GAS positive cells could be seen, virtually all being of the small type. No met ENK immunoreactive cells could be demonstrated in the ganglion. In the first sacral spinal ganglion numerous GAS positive neurons, partly of the large type were seen. In addition SP, SOM and VIP but no ENK immunoreactive small neurons were observed.

The present results indicate that in the vagus nerve complex as well as in the sciatic nerve SP, SOM (CCK) like peptides, at least partly are present in sensory neurons, whereas a

TABLE I. Subjective estimation of the number of peptide containing axons in the vagus, splanchnic and sciatic nerve of the guinea pig. The estimates are based on analysis of accumulations of immunoreactive material above ligation: + + + + + = very high, + + + + = high, + + + = medium, + + = low and + = single. LI = Like immunoreactivity. For abbreviations of peptides see text.

	SP-LI	SOM-LI	VIP-LI	GAS-(CCK)-LI	met ENK-LI
N vagus	+ + + + +	+	+ + +	+	+ +
N splanchnicus major	+ + +	+	+	+	+ + +
N behadicus	+ + +	+	+ +	+ + +	+

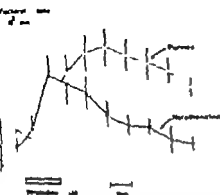


Fig. 1



Fig. 2

1. Release of ^3H -labelled purines and ^3H -labelled noradrenaline from prelabeled rat cortical slices. The data are given as fractional release per min and represent mean \pm S.E. of 5 experiments.

2. Effect of morphine (M), naloxone (N) and the combination on the veratridine induced purine release from cortical slices. The drugs were present at $1 \mu\text{M}$ concentration between 65 and 100 min after the start of superfusion. Veratridine was present in the superfusion period at 47-50 min (S_1) and at 81-90 min (S_2). The data are given as S_2/S_1 ratios of individual experiments. Mean \pm S.E. of 6 experiments.

After the slices were removed and degraded in HCl tissue solubilizer (Amersham, G.B.) and the radioactivity counted. The fractional release is the amount released in each collected fraction divided by the amount at the time of collection) as calculated.

After some 30 min the rate of efflux of purines reached a steady level of 0.71 ± 0.01 (μM) out of tissue content per min and after 70 min it was 0.74 ± 0.11 . The basal rate of release was not affected by $1 \mu\text{M}$ morphine (0.78 ± 0.09), $1 \mu\text{M}$ naloxone (0.71 ± 0.09) or by $1 \mu\text{M}$ veratridine (0.72 ± 0.1). Between 7 and 50 min after start of superfusion veratridine ($1 \mu\text{M}$) was included in the buffer. As seen in Fig. 1 this caused the release of purines as well as noradrenaline. The release of noradrenaline was abolished by omission of calcium and inclusion of EGTA, while the purine release was more than 50% reduced. The purine compounds were inosine, adenosine and hypoxanthine predominantly as judged by TLC. Although the release of noradrenaline is much faster in onset than the release of purines (Fig. 1). This could indicate that the mechanism behind the release of the two types of compounds were different, for example that purine release is secondary to the release of a very transmitter.

The depolarization-induced release of purines was not completely reproducible. During the first stimulation (47-50 min) 3.4 ± 0.4 of tissue content was released during the second (51-60 min) 2.2 ± 0.7 . Therefore the effect of drugs was tested during the second stimulation period (present from 65 min onwards) and the amount released during this second stimulation (S_2) was divided by that released during the first (S_1) in the same slices. It is seen in Fig. 2 that morphine ($1 \mu\text{M}$) caused a significant enhancement of the purine release. Naloxone ($1 \mu\text{M}$) had no effect, nor did the combination of morphine and naloxone. Thus, the present results demonstrate that morphine increases veratridine induced purine release from rat cortical slices. The effect of morphine was not observed in the presence of

Morphine increases depolarization induced purine release from rat cortical slices

By

BERTIL B. FREDHOLM and LOUISE VERNET

Theophylline antagonizes several different actions of morphine, including analgesia *in vivo* (Ho *et al.* 1973), the inhibitory effect of morphine on electrically induced contractions in the guinea pig longitudinal muscle myenteric plexus (Sawynok and Jhamandas 1976), the direct stimulatory effect of morphine (Grubb and Burks 1975), and the inhibitory effect of morphine on acetylcholine from brain slices (Jhamandas *et al.* 1978). The mechanism of action of theophylline is not known. However, theophylline is known to antagonize several actions of adenosine. For example, the inhibitory effect of adenosine on field stimulated guinea-pig longitudinal muscle is inhibited by theophylline (Sawynok and Jhamandas 1976). Adenosine inhibits acetylcholine and noradrenaline release (Sawynok and Jhamandas 1976, Vizi and Knoll 1976) and this effect is also antagonized by theophylline.

Theophylline thus inhibits effects of both morphine and adenosine, but the two types of agents are not acting on the same receptors, since adenosine effects are not antagonized by naloxone which readily inhibits morphine effects (Sawynok and Jhamandas 1976, Gustafsson, Hedqvist and Fredholm, unpublished data). The data are, however, compatible with the opinion that morphine induces release of adenosine (or a related compound) and that this accounts at least partly for the effects of morphine. Circumstantial support for such a scheme is provided by the report that the inhibitory effect of morphine on contractions of the field stimulated guinea pig ileum is potentiated by dipyrindamol (Gintzler and Musacchio 1975), a compound known to potentiate the effects of adenosine by inhibiting its inactivation.

We report the preliminary finding that morphine enhances depolarization induced release of adenosine and other purines from rat cortical slices.

After decapitation the rat brains were rapidly dissected out. Thin slices (0.5-1 mm) of the occipital cortex were cut by a razor blade. From the middle of the slice, a circular section (7 mm diam) was punched out. 4 slices were incubated at 37°C in 5 ml Krebs-Ringer bicarbonate buffer for 40 min with 10 μ M 2,8-³H-adenine, ¹⁴C-adenine or 1-7 ³H-noradrenaline (Radiochemical Centre, Amersham). On the average some 10 nCi was taken up per slice. After washing, the slices were placed in thermostated plastic chambers and superfused with Krebs-Ringer bicarbonate buffer of the following composition (mM): NaCl (118), KCl (4.85), CaCl₂ (0.25), + MgSO₄ (1.15), KH₂PO₄ (1.15), NaHCO₃ (25), glucose (11.1), pH 7.2-7.4 gassed with 95% O₂ and 5% CO₂.

Samples of the superfusion solution were continuously taken and the radioactivity counted in scintillation cocktail consisting of 4 g/l Omnifluor (Packard) in toluene-Triton X 100 (2:1). At the end of the ex-

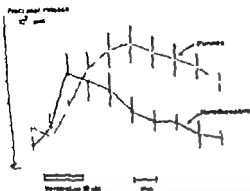


Fig. 1

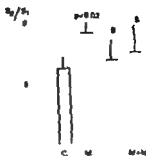


Fig. 2

Fig. 1 Release of ^3H -labelled purines and ^3H -labelled noradrenaline from prelabeled rat cortical slices. Results are given as fractional release per min and represent mean \pm S.E. of 5 expts.

Fig. 2 Effect of morphine (M), naloxone (N) and the combination on the veratridine induced purine release from cortical slices. The drugs are present at $1 \mu\text{M}$ concentrations between 45 and 106 min after the start of superfusion. Veratridine is present in the superfusion period at 47-90 min (S_2) and at 87-90 min (S_1). Results are given as S_2/S_1 ratios of individual experiments. Mean \pm S.E. of 6 expts.

permeant the slices were removed and digested in NCS tissue solubilizer (Amersham, G.B.) and in radioactivity counter. The fractional release (i.e. the amount released in each collected fraction divided by the tissue content at the time of collection) was calculated.

After some 30 min the rate of efflux of purines reached a steady level of 0.71 ± 0.03 (24) per cent of tissue content per min and after 70 min it was 0.74 ± 0.11 . The basal rate of efflux was not affected by $1 \mu\text{M}$ morphine (0.78 ± 0.09), $1 \mu\text{M}$ naloxone (0.71 ± 0.09) or by morphine-naloxone (0.82 ± 0.12). Between 7 and 30 min after start of superfusion veratridine ($10 \mu\text{M}$) was included in the buffer. As seen in Fig. 1 this caused the release of purines as well as noradrenaline. The release of noradrenaline was abolished by omission of calcium and inclusion of EGTA, while the purine release was more than 50% reduced. The purine compounds were inosine, adenosine and hypoxanthine, predominantly as judged by TLC. Interestingly the release of noradrenaline was much faster in onset than the release of purines (Fig. 1). This could indicate that the mechanism behind the release of the two types of compounds were different, for example that purine release is secondary to the release of a primary transmitter.

The depolarization-induced release of purines was not completely reproducible. During the first stimulation (47-90 min) $3.4 \pm 0.4\%$ of tissue content was released, during the second (87-90 min) 2.2 ± 0.7 . Therefore the effect of drugs was tested during the second stimulation period (present from 65 min onwards) and the amount released during this second stimulation (S_2) was divided by that released during the first (S_1) in the same slice. It is seen in Fig. 2 that morphine ($1 \mu\text{M}$) caused a significant enhancement of the purine release. Naloxone ($1 \mu\text{M}$) had no effect, nor did the combination of morphine and naloxone.

Thus, the present results demonstrate that morphine increases veratridine induced purine release from rat cortical slices. The effect of morphine was not observed in the presence of

Morphine increases depolarization induced purine release from rat cortical slices

By

BERTIL B. FREDHOLM and LOUISE VERNET

Theophylline antagonizes several different actions of morphine, including analgesia in mice (Ho *et al* 1973) the inhibitory effect of morphine on electrically induced contractions in the guinea pig longitudinal muscle myenteric plexus (Sawynok and Jhamandas 1976), the direct stimulatory effect of morphine (Grubb and Burks 1975), and the inhibitory effect of morphine on acetylcholine from brain slices (Jhamandas *et al* 1978). The mechanism of action of theophylline is not known. However theophylline is known to antagonize several actions of adenosine. For example, the inhibitory effect of adenosine on field stimulated guinea-pig longitudinal muscle is inhibited by theophylline (Sawynok and Jhamandas 1976). Adenosine inhibits acetylcholine and noradrenaline release (Sawynok and Jhamandas 1976, Vizi and Knoll 1976 and this effect is also antagonized by theophylline.

Theophylline thus inhibits effects of both morphine and adenosine, but the two types of agents are not acting on the same receptors, since adenosine effects are not antagonized by naloxone which readily inhibits morphine effects (Sawynok and Jhamandas 1976, Gustafsson, Hedqvist and Fredholm, unpublished data). The data are, however compatible with the opinion that morphine induces release of adenosine (or a related compound) and that this accounts at least partly for the effects of morphine. Circumstantial support for such a scheme is provided by the report that the inhibitory effect of morphine on contractions of the field stimulated guinea-pig ileum is potentiated by dipyrndamol (Gintzler and Musacchio 1975), a compound known to potentiate the effects of adenosine by inhibiting its inactivation.

We report the preliminary finding that morphine enhances depolarization induced release of adenosine and other purines from rat cortical slices.

After decapitation the rat brains were rapidly dissected out. Thin slices (0.5-1 mm) of the occipital cortex were cut by a razor blade. From the middle of the slice a reticular section (7 mm diam) was punched out. 4 slices were incubated at 37°C in 5 ml Krebs-Ringer bicarbonate buffer for 40 min with 10 μ M 2,8-³H-adenosine. ³H-adenosine or 1-7 ³H-noradrenaline (Radiochemical Centre, Amersham). In the average some 10 nCi was taken up per slice. After washing, the slices were placed in thermostated plastic chambers and superfused with Krebs-Ringer bicarbonate buffer of the following composition (mM): NaCl (118), KCl (4.85), CaCl₂ (0.25), + MgSO₄ (1.15), KH₂PO₄ (1.15), HCO₃⁻ (25) glucose (11.1), pH 7.2-7.4 gassed with 95% O₂ and 5% CO₂.

Samples of the superfusion solution were continuously taken and the radioactivity counted in a scintillation cocktail consisting of 4 g/l Omalfloor (Packard) in toluene Triton X 100 (1:1). At the end of the ex-

1 Communications.

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naloxone suggesting that it is exerted over morphine receptors. The mechanism behind the veratridine-induced release of purines is not known, therefore the mechanism behind the morphine effect remains obscure. Veratridine is known to depolarize nervous elements causing rapid changes in ion composition including a rise in internal calcium level (Blaustein 1975). There is also a rapid fall in ATP content (Li and White 1976). The release of purines may be a reflection of this fall in ATP content, and morphine may enhance purine release by enhancing ATP breakdown or diminishing its resynthesis. Morphine did not enhance spontaneous overflow indicating that whatever the mechanism may prove to be it would appear to be dependent upon nerve activity leading to depolarization.

Although the data are very preliminary it is interesting to speculate that increased levels of extracellular purines may contribute to the effects of morphine. As noted in the introduction this may possibly explain the otherwise puzzling partial morphine antagonism by methylxanthines. In view of the manifold effects of purines on nervous tissue, including increased cyclic AMP levels, decreased neuronal firing rate, and decreased transmitter release (Phillis 1977, Hedqvist and Fredholm 1976, Vizi and Knoll 1976) further studies along these lines are being carried out.

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SI units with recommended symbols

Units	Symbols
kilogramme	kg
second, millisecond	s ms
mole, millimole, micro-mole, nanomole, picomole	mol mmol μ mol nmol pmol
meter millimeter micrometer nanometer	m mm μ m nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N ($kg\ m/s^2$)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J ($N\ m$)
watt (effect)	W (J/s)
himen (light flow)	lm ($cd\ sr$)
lux (illumination)	lx (lm/m^2)

Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)
curie	Cl
liter milliliter microliter	l ml μ l
degree Celsius	$^{\circ}C$

Conversion factors to be given in Methods.

